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(54) Title: SECRETASES RELATED TO ALZHEIMER'S DEMENTIA (54) Titre: SECRETASES LIEES A LA DEMENCE D'ALZHEIMERA (57) Abstract <p>The invention is directed to methods of selecting secretases that agents that cleave the amyloid precursor protein (APP) substrate, inhibiting production of the A'beta' peptide found in Alzheimer's Disease and treating Alzheimer's disease in patients. The invention also is directed to a novel 'beta'-secretase that produces the A'beta' peptide. One 'beta'-secretase is a protein having a molecular weight of about 61, 81 or 88 kDa that cleaves an. Another is a protease complex having a molecular weight between about 180 and 200 kDa, which, in one embodiment, contains the 61, 81, and 88 kDa proteins and, in another embodiment, contains proteins having a molecular weight of about 66, 60, 33 and 29 kDa. Another 'beta'-secretase has a molecular weight between about 50 and 90 kDa.</p> (57) Abrégé <p>L'invention a trait à des procédés de sélection de sécrétases qui coupent le substrat de la protéine précurseur amyloïde (APP), inhibant ainsi la production du peptide A'beta' présent dans la maladie d'Alzheimer et permettant de traiter des patients atteints de cette maladie. L'invention a également trait à une nouvelle 'beta'-sécrétase qui produit le peptide A'beta'. Une 'beta'-sécrétase est une protéine présentant un poids moléculaire d'environ 61, 81 ou 88 kDa. Une autre sécrétase est un complexe de protéases présentant un poids moléculaire se situant entre environ 180 et 200 kDa, et qui, dans un mode de réalisation, contient les protéines de 61, 81 et 88 kDa, et, dans un autre mode de réalisation, contient des protéines présentant un poids moléculaire d'environ 66, 60, 33 and 29 kDa. Une autre 'beta'-sécrétase présente un poids moléculaire se situant entre environ 50 et 90 kDa.</p>		

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(54) Title: SECRETASES RELATED TO ALZHEIMER'S DEMENTIA			
(57) Abstract <p>The invention is directed to methods of selecting secretases that agents that cleave the amyloid precursor protein (APP) substrate. Inhibiting production of the Aβ peptide found in Alzheimer's Disease and treating Alzheimer's disease in patients. The invention also is directed to a novel β-secretase that produces the Aβ peptide. One β-secretase is a protein having a molecular weight of about 61, 81 or 88 kDa that cleaves an. Another is a protease complex having a molecular between about 180 and 200 kDa, which, in one embodiment, contains the 61, 81, and 88 kDa proteins and, in another embodiment, contains proteins having a molecular weight of about 66, 60, 33 and 29 kDa. Another β-secretase has a molecular weight between about 50 and 90 kDa.</p>			

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Description

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SECRETASES RELATED TO ALZHEIMER'S DEMENTIA

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This invention was made with United States government support under grant number NS24553 awarded by the National Institutes of Neurological Disease and Stroke. The United States government has certain rights in the invention.

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BACKGROUND OF THE INVENTION

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FIELD OF THE INVENTION

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The present invention relates generally to medicine. More specifically, the invention is directed to methods relating to treating or preventing dementia.

BACKGROUND INFORMATION

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Dementia is a neurological disease that results in loss of intellectual capacity and is associated with widespread reduction in the number of nerve cells and brain tissue shrinkage. Memory is the mental capacity most often affected. The memory loss may first manifest itself in simple absentmindedness, a tendency to forget or misplace things, or to repeat oneself in conversation. As the dementia progresses, the loss of memory broadens in scope until the patient can no longer remember basic social and survival skills and function independently. Dementia can also result in a decline in the patient's language skills, spatial or temporal orientation, judgment, or other cognitive capacities. Dementia tends to run an insidious and progressive course.

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Dementia results from a wide variety of distinctive pathological processes. The most common pathological process to cause dementia is Alzheimer's disease, which results in Alzheimer's-type dementia (AD). The second most common cause is multi-infarct, or vascular dementia, which results from hypertension or other vascular conditions. Dementia can also result from infectious disease, such as in Creutzfeldt-Jakob disease. Dementia occurs in Huntington's disease, which is caused by an autosomal dominant gene mutation, and in Parkinson's disease, which is associated with a motor disorder. Dementia also occurs from head injury and tumors.

Rare before age 50, AD affects nearly half of all people past the age of 85, which is the most rapidly growing portion of the United States population. As such, the current 4 million AD patients in the United States are expected to increase to about 14 million by the middle of the next century.

No method of preventing AD is known and treatment is primarily supportive, such as that provided by a family member in attendance. Stimulated memory exercises on a regular basis have been shown to slow, but not stop, memory loss. A few drugs, such as tacrine, result in a modest temporary improvement of cognition but do not stop the progression of dementia.

A hallmark of AD is the accumulation in brain of extracellular insoluble deposits called amyloid plaques, and abnormal lesions within neuronal cells called neurofibrillary tangles. The presence of amyloid plaques, together with neurofibrillary tangles, are the basis for definitive pathological diagnosis of AD.

Increased plaque formation is associated with increased risk of AD.

The major components of amyloid plaques are the amyloid β -peptides, also called A β peptides, which consist of three proteins having 40, 42 or 43 amino acids, designated as the A β_{1-40} , A β_{1-42} , and A β_{1-43} peptides. The amino acid sequences of the A β peptides are known and the sequence of the A β_{1-42} is identical to that of the A β_{1-40} peptide, except that the A β_{1-42} peptide contains two additional amino acids at its carboxyl (COOH) terminus. Similarly, the amino acid sequence of the A β_{1-43} peptide is identical to that of the A β_{1-42} peptide except that the A β_{1-43} peptide contains one additional amino acid at its carboxyl terminus. The A β peptides are thought to cause the nerve cell destruction in AD, in part, because they are toxic to neurons *in vitro* and *in vivo*.

The A β peptides are derived from larger amyloid precursor proteins (APP proteins), which consist of four proteins, designated as the APP₆₉₅, APP₇₁₄, APP₇₅₁, and APP₇₇₁ proteins, which contain 695, 714, 751 or 771 amino acids, respectively. The different APP proteins result from alternative ribonucleic acid splicing of a single APP gene product. The amino acid sequences of the APP proteins are also known and each APP protein contains the amino acid sequences of the A β peptides.

Proteases are believed to produce the A β peptides by recognizing and cleaving specific amino acid sequences within the APP proteins at or near the ends of the A β peptides. Such sequence specific proteases are thought to exist because they are necessary to produce from the APP proteins the A β_{1-40} , A β_{1-42} , and A β_{1-43} peptides consistently found in plaques.

5 But the proteases have not been isolated.
Nonetheless, they have been named "secretases" because
the A β peptides which they produce are secreted by cells
10 into the extracellular environment. Moreover, the
5 secretases have been named according to the cleavages
that must occur to produce the A β peptides. The
secretase that cleaves the amino terminal end of the A β
15 peptides is called the β -secretase and that which cleaves
the carboxyl terminal end of the A β peptides is called
10 the γ -secretase. The γ -secretase determines whether the
A β_{1-40} , A β_{1-42} , or A β_{1-43} peptide is produced (see Figure 1).
20 But since the secretases have not been isolated, the
terms β -secretase and γ -secretase each could relate to
one or several protease species.

25 15 In addition to the A β peptides, proteolytic
cleavage of another specific amino acid sequence within
the APP proteins is known to occur and to produce α -APP
and 10 kilodalton (kDa) fragments. That amino acid
30 sequence lies within the A β peptide amino acid sequence
of the APP proteins. Like the β -secretase and the
20 γ -secretase, the protease responsible for that cleavage
has also not been isolated but has been named the
35 α -secretase (see Figure 1). Significantly, the products
produced by the α -secretase cleavage, the α -APP and the
25 10 kilodalton (kDa) fragments, do not form senile
40 plaques.

Proteases can be isolated from tissue
homogenates or lysed cell samples, but those samples can
45 contain the proteases from cell organelles in which the
product is not produced, but which may be able to cleave
30 in vitro the precursor protein to produce the product.
Thus, a problem in using such samples to isolate the
50 secretases has been that proteases which produce the A β

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peptide *in vitro*, but not *in vivo*, may be erroneously isolated.

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The problem can be avoided by isolating the secretase from cell organelles in which the APP proteins are processed *in vivo*. A cell organelle thought to be a site in which such processing occurs is the secretory vesicles of brain neuronal cells. But methods have not been developed to obtain sufficient amounts of pure secretory vesicles from neuronal cells to assay for secretase activity in those vesicles.

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Large amounts of pure secretory vesicles can be obtained from chromaffin cells, neuroendocrine cells of the adrenal medulla, and have been used to obtain proteases. For example, carboxypeptidase H (CPH), prohormone thiol protease (PTP), and the prohormone convertases (PC1 and PC2), which process precursor proteins into peptides having opiate activity have been obtained from such vesicles. But chromaffin cells have not been shown to produce the A β peptides or have secretase activity.

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The β -secretase, γ -secretase, and α -secretase must be isolated to understand how the neurotoxic A β peptides are produced so that AD can be prevented or treated. To isolate the secretase, new methods are needed for assaying the proteolytic activity of secretases in substantially purified preparations of the cell organelles in which the APP protein is processed *in vivo*. Moreover, new screening methods for selecting agents that affect the proteolytic activity of the secretases are needed to develop new pharmaceuticals for treating or preventing AD. Further, such new methods need to be applied and the secretases isolated.

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The invention satisfies these needs by providing new methods of determining the proteolytic activity of secretases and isolating secretases having that activity. The invention also provides new screening methods for selecting agents that affect the activity of such secretases. Moreover, the invention discloses novel β -secretases obtained by such methods as well as methods of selecting agents inhibiting production of A β peptides by inhibiting the activity of those β -secretases.

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SUMMARY OF THE INVENTION

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The invention is directed to various novel β -secretases. One such β -secretase contains a protein having a molecular weight of about 61, 81 or 88 kDa as determined by cleavage of an APP substrate in a non-reducing SDS-PAGE in gel activity assay. In one embodiment, the β -secretase contains a protein that cleaves the APP substrate in the β -secretase recognition site at the Lys-Met bond.

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Another is a protease complex having a molecular weight between about 180 and 200 kiloDaltons (kDa) as determined by Sephacryl chromatography that cleaves an APP substrate. In one embodiment, the protease complex cleaves the APP substrate in the β -secretase recognition site at the Lys-Met bond. In another embodiment, the protease complex contains proteins having molecular weights of about 66, 60, 33 and 29 kDa as determined by a reducing SDS-PAGE in gel protein staining assay. In another embodiment, the protease complex contains proteins having molecular weights of about 61, 81 and 88 kDa as determined by cleavage of an APP substrate in a non-reducing SDS-PAGE in gel activity assay.

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Another β -secretase has a molecular weight between about 50 and 90 kDA as determined by Sephacryl chromatography and cleaves an APP substrate. In one embodiment, that β -secretase cleaves the APP substrate in the β -secretase recognition site at the Met-Asp bond. In another embodiment, the β -secretase contains 2 proteins having different electronegative charges as determined by ion exchange chromatography.

The invention is also directed to a method of selecting an agent that inhibits cleavage of the APP substrate by the β -secretases described above. The invention is further directed to a method of inhibiting production of an A β peptide by a cell or by an Alzheimer's disease patient using such a selected agent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. The upper bar is a diagram of an amyloid precursor protein (APP protein). The amino and carboxyl termini of the APP protein are indicated by the letters "N" and "C," respectively. The relative location of various known regions within the APP protein are indicated, including the signal peptide (SP), cysteine-rich (C-rich), negatively charged ((-)charged), protease inhibitor, Ox antigen (Ox), transmembrane, cytoplasmic and A β peptide regions. The amino acid sequence of the A β peptides and regions flanking the A β peptides is shown by the letters below the amyloid precursor protein (SEQ ID NO.:1). Each letter represents an amino acid according to the conventional single letter amino acid abbreviation format. Scissile bonds within the amino acid sequence cleaved by the β -, γ -, or α -secretases are indicated by the β , γ , and α labels. Three scissile bonds cleaved by β -secretases which, in

5 combination with scissile bond cleaved by the
γ-secretase, produce the Aβ₁₋₄₀, Aβ₁₋₄₂, or Aβ₁₋₄₃ peptide.
The three parallel lines below the amino acid sequence
10 identify the amino acid sequences of the Aβ₁₋₄₀, Aβ₁₋₄₂, and
5 Aβ₁₋₄₃ peptides.

Figure 2. The bonds, labeled #1, #2, and #3,
15 in the Z*Val-Lys-Met-MCA substrate cleaved by a secretase
having endoprotease activity are shown. The Z, Val, Lys,
Met, and MCA in the substrate represent a carbobenzoxy,
10 valine, lysine, methionine, and aminomethylcoumarinamide
20 group, respectively. The star (*) and dash (-) represent
nonpeptide and peptide bonds, respectively

Figure 3. The fluorescence activity is plotted
25 as a function of the pH at which a lysate of
15 substantially pure chromaffin vesicles is incubated with
the Z*Val-Lys-Met-MCA substrate. The fluorescence
activity is the relative fluorescence of the free MCA
30 (AMC) released by proteolytic cleavage of the substrate.

Figure 4. The fluorescence activity is
35 plotted as a function of the pH at which a lysate of
20 substantially pure chromaffin vesicles is incubated with
the Met-MCA substrate. The fluorescence activity is the
relative fluorescence of the free MCA (AMC) released by
40 proteolytic cleavage of the substrate.

Figure 5. The fluorescence activity is plotted
25 as a function of the pH at which the lysate of
45 substantially pure chromaffin vesicles is incubated with
the Lys-MCA substrate. The fluorescence activity is the
relative fluorescence of the free MCA (AMC) released by
30 proteolytic cleavage of the substrate.

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Figure 6. The fluorescence activity is plotted as a function of the pH at which the lysate of substantially pure chromaffin vesicles is incubated with the Z*Val-Lys-Met-MCA substrate in the presence and absence of DTT (closed and open squares, respectively). The fluorescence activity is the relative fluorescence of the free MCA (AMC) released by proteolytic cleavage of the substrate.

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Figure 7. The fluorescence activity is plotted as a function of the pH at which the lysate of substantially pure chromaffin vesicles is incubated with the Z*Val-Lys-Met-MCA substrate in the presence of DTT without aminopeptidase M (open triangles), with basic pH buffer (open squares), or with aminopeptidase M (closed squares). The fluorescence activity is the relative fluorescence of the free MCA (AMC) released by proteolytic cleavage of the substrate.

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Figures 8. The isolation procedure used to obtain Peak I and Peak II is diagramed.

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Figure 9. The fluorescence activity is plotted as a function of the fraction number (#) obtained from the Sephacryl S200 in the procedure diagramed in Figure 8. Fraction numbers 30 to 40, and 40 to 50 contain Peak I and Peak II, respectively. The activity is that which results from cleavage of the Z*Val-Lys-Met-MCA substrate by the fraction without aminopeptidase M (open squares), or with aminopeptidase M (closed squares). The fluorescence activity is in pmol of free MCA per microliter (AMC/ μ l). The γ -globulin, BSA, and myoglobin are calibration weight standards.

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Figure 10. The procedure used to isolate the β -secretases from Peak I is diagramed.

Figure 11. The procedure used to isolate the β -secretases from Peak II is diagramed.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides an assay for the proteolytic activity of secretases, particularly the β -secretase and the γ -secretase that produce the A β peptides found in the plaques of AD patients. The method is novel because the activity is detected in a substantially purified preparation of vesicles in which APP protein processing occurs *in vivo*. Based on that activity assay, new methods are disclosed to isolate the secretases from such substantially purified preparations. Isolating the secretases from the cell organelles in which the APP protein is processed insures that the secretases are the *in vivo* secretases and not merely a protease from a cell organelle in which such processing does not occur, but which is capable of cleaving the APP protein *in vitro*. The invention further provides methods of selecting an agent that affects the proteolytic activity of the substantially purified vesicles, the isolated secretase, or the cells containing the vesicles.

As discussed in Examples V and VII below, the secretory vesicles of chromaffin cells of the adrenal medulla, herein called "chromaffin vesicles," were discovered to contain A β peptides, specifically the A β_{1-40} and the A β_{1-42} peptides, and that chromaffin cells can secrete these peptides. As such, the chromaffin vesicles were found to contain the *in vivo* product produced by APP protein processing. Moreover, the vesicles were known to

5 contain the APP proteins and presenilin 1 protein, a
protein that affects secretase activity (see
Vassilacopoulou et al., *J. Neurochem.* 64:2140-2146,
10 (1995); Tezapsidis et al., *Biochem.* 37(5):1274-1282,
5 (1998); Borchelt et al., *Neuron* 17:1005-1013, (1996); St.
George-Hyslop et al., *Science* 264:1336-1340, (1994);
Alzheimer's Disease Collaborative Group, *Nature Genet.*
15 11:219-222, (1995); and Wasco et al., *Nature Med.* 1:848,
(1995)).

20 10 Chromaffin vesicles can be obtained in
relatively large quantities. That capability, combined
with the discovery that the chromaffin vesicles contained
the A β peptides, permitted for the first time assaying a
25 substantially pure preparation of cell organelles in
15 which APP processing occurs for the proteolytic activity
of a secretase. Further, chromaffin vesicles can be
obtained in amounts which also permit isolating and
30 sequencing the secretases present in those cell
organelles.

35 20 As described more fully below in Examples I
through XV, bovine chromaffin vesicles were initially
discovered to have secretase proteolytic activity.
Moreover, it was found that secretases having that
40 activity could be isolated from bovine chromaffin
25 vesicles. But the same methods can be applied to other
mammalian species, including humans. As such, secretases
from various mammalian species can be assayed for and
isolated using the methods disclosed herein.

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30 Further, the amino acid sequence of a bovine
secretase is likely to be highly homologous with that of
50 the corresponding human secretase because other bovine
proteases are known to have a high degree of homology

5 with the corresponding human protease. For example, the
amino acid sequence of the bovine carboxypeptidase H is
about 96% homologous with the corresponding human
10 carboxypeptidase H (Hook et al., *Nature*, 295:341-342,
5 (1982); Fricker et al., *Nature*, 323:461-464, (1986); and
Manser et al., *Biochem. J.*, 267:517-525, (1990)). Once
the amino acid sequence of a secretase from one species
15 is obtained, the corresponding secretase in other species
thus can be obtained using recombinant methods such as
10 those described below.

20 The term "secretase" as used herein means a
protease that cleaves an APP protein in vivo. A protease
is a protein that enzymatically breaks a peptide bond
between two amino acids or an amino acid and chemical
25 moiety as described below. Although the term secretase
15 implies the production of a soluble, secreted peptide, an
APP derived product produced by a secretase of the
invention need not necessarily be soluble or secreted.
30 "Secretase" includes those secretases referred to as
20 β -secretase and γ -secretase, each of which can relate to
one or more protease species that produce the A β
peptides. "Secretase" also includes the secretase
35 referred to as α -secretase which can relate to one or
more protease species that produce the α -APP fragment or
25 the 10 kDa fragment.

40 The term "vesicles" as used herein refers to
secretory vesicles and condensing vacuoles of the
secretory pathway. Such vesicles have a membrane that
45 forms a spherical shaped structure and that separates
30 the contents of the vesicles from the rest of the cell.
The vesicles process and store their contents until such
time as the contents are secreted into the extracellular
50 environment by a cellular process called exocytosis.

5 which occurs by fusion of the secretory vesicle membrane
with the cell membrane. The secretion can occur in
response to a triggering event in the cell such as a
10 hormone binding to a receptor. Vesicles can be
5 identified by their characteristic morphology or by the
presence of a chemical compound characteristic of such
vesicles.

15 As used herein, the term "substantially pure"
as used in regard to vesicles means that at least about
10 80% of the cell organelles in a sample are vesicles.
20 Usually a substantially pure sample has about 95% or more
vesicles and often has about 99% or more vesicles.
Substantially pure vesicles include a single isolated
vesicle. Substantially pure chromaffin vesicles result
25 after approximately an 8-fold purification from the cell
homogenate as described below in Example II.

30 METHODS OF DETERMINING THE PROTEOLYTIC ACTIVITY OF A SECRETASE

35 One aspect of the invention is an assay for
20 determining the proteolytic activity of a secretase by
obtaining substantially pure vesicles, permeabilizing the
vesicles, and incubating the permeabilized vesicles with
an APP substrate in conditions which allow the secretase
40 to cleave the APP substrate. The cleavage of the APP
25 substrate is detected and the activity of the secretase
is thereby determined.

45 The vesicles can be obtained from any cell that
contains vesicles in which APP protein processing occurs.
Vesicles in which such processing occurs can be assayed
30 for by the presence of an A β peptide, an α -APP fragment
50 or a 10 kDa fragment in the vesicles using methods

described below. Cells containing such vesicles include, for example, neuronal cells from brain tissue, chromaffin cells from adrenal medulla tissue, and platelets from blood. Tissue samples containing such cells can be surgically removed or platelets can be isolated from blood by means known in the art. For tissue samples, the vesicles can be obtained from mechanically homogenized tissue or from tissue disassociated by incubation with collagenase and DNase (see, for example, Krieger et al., *Biochemistry*, 31, 4223-4231, (1992); Hook et al., *J. Biol. Chem.*, 260:5991-5997, (1985); and Tezapsidis et al., *J. Biol. Chem.*, 270:13285-13290, (1995), which are incorporated herein by reference).

The substantially pure vesicles can be obtained from the tissue homogenates or lysed cells using known methods (see *Current Protocols in Protein Science*, Vol. 1 and 2, Coligan et al., Ed., John Wiley and Sons, Pub., Chapter 4, pp. 4.0.1-4.3.21, (1997)). For example, substantially pure secretory vesicles can be isolated using discontinuous sucrose gradient centrifugation methods (see Krieger et al., *ibid.*; and Yasothornsrikul et al., *J. Neurochem.* 70, 153-163, (1998)). Vesicles also can be isolated using metrizamide gradient centrifugation (Toomin et al., *Biochem. Biophys. Res. Commun.*, 183:449-455, (1992); and Loh et al., *J. Biol. Chem.*, 259:8238-8245, (1984), or percoll gradient centrifugation (Russell, *Anal. Biochem.*, 113:229-238, (1981). If desired, capillary electrophoresis methods can be used to isolate individual vesicles (Chie et al., *Science*, 279:1190-1193, (1998)). Other methods, including differential centrifugation, fluorescence-activated sorting of organelles, immunocapture isolation, elutriation centrifugation,

5 gel filtration, magnetic affinity chromatography, protein
chromatographic resins, agarose gel electrophoresis, and
free flow electrophoresis methods, also can be used to
10 obtain substantially pure vesicles. The references cited
5 in this paragraph are incorporated by reference.

The purity of the secretory vesicle preparation
15 can be assayed for by morphological or chemical means.
For example, vesicles can be identified by their
characteristic morphology as observed by electron
10 microscopy. The vesicles can be prepared for electron
20 microscopy using various methods including ultra-thin
sectioning and freeze-fracture methods. Vesicles also
can be identified by the presence of a characteristic
neurotransmitter or hormone present in such vesicles such
25 as the (Met)enkephalin, catecholamines, chromogranins,
15 neuropeptide Y, vasoactive intestinal peptide,
somatostatin, and galanin found in chromaffin vesicles
(Hook and Eiden, *FEBS Lett.* 172:212-218, (1984); Loh et
30 al., *J. Biol. Chem.* 259:8238-8245, (1984);
20 Yasothornsrikul et al., *J. Neurochem.* 70:153-163, (1998),
which are incorporated herein by reference). The
35 presence of the characteristic chemical compound can be
determined by various means including, for example, by
radioactive, fluorescent, cytochemical, immunological
25 assays, or mass spectrometry methods. More specifically,
40 such assays include radioimmunoassays, western blots or
MALDI mass spectrometry. In addition, vesicles can be
assayed using light and electron microscopic methods,
fluorescent cell activated cell sorter methods, density
45 30 gradient fractionation methods, immunoabsorption methods,
or biochemical methods.

The activity of the secretases can be preserved
50 while the vesicles are purified using known methods. For

5 example, the vesicles can be obtained at a low
temperature (e.g. 4°C) and frozen (e.g. -70°C) prior to
assaying for secretase activity. The activity can also
10 be preserved by obtaining the vesicles in the presence of
5 a stabilizing agent known to preserve protease activity
(see *Enzymes*, Dixon et al., Eds., Academic Press, Pub.,
pp. 11-12, (1979), and *Current Protocols in Protein*
15 *Science*, , Vol. 1 and 2, Coligan et al., Ed., John Wiley
and Sons, Pub., Chapter 4, pp. 4.5.1-4.5.36, (1997),
10 which are incorporated herein by reference). Known
stabilizing agents include proteins, detergents and
20 salts, such as albumin protein, CHAPS, EDTA, glycerol,
and NaCl. Reducing agents are also known to preserve
protein function and can be used (see Voet et al.,
15 *Biochemistry*, John Wiley and Sons, Pub., pp. 382-388 and
750-755, (1990), which is incorporated herein by
reference). Known reducing agents include, for example,
β-mercaptoethanol, DTT, and reduced glutathione (see
30 Example VIII).

20 So that secretases within the vesicles are
accessible to an APP substrate in an incubation solution,
35 the vesicles are permeablized (see Voet et al.,
Biochemistry, John Wiley and Sons, Pub., pp. 284-288,
(1990); and Krieger et al., *ibid.*, which are incorporated
25 herein by reference). Permeabilizing can result in a
40 continuum of affects on the vesicle ranging from the
formation of one or more holes in the membrane to
complete lysis of the membrane. Vesicles can be
permeablized, for example, by contact with a detergent or
45 30 a disruptive agent such as CHAPS, sodium dodecyl sulfate,
sodium cholate, digitonin, Brij 30 or TRITON X-100.
Vesicles can be lysed, for example, by freeze-thawing,
50 especially in a potassium chloride solution, by

5 suspension in a hypoosmotic solution or by mechanical means such as sonication.

10 The permeablized vesicles are incubated with an APP substrate under appropriate conditions for cleavage
5 of the APP substrate by a secretase. Various incubation conditions are known to affect protease cleavage. For
15 example, the pH of the interior of chromaffin vesicles is acidic and some proteases in those vesicles are known to only function in an acidic incubation solution (Pollard
10 et al., *J. Biol. Chem.* 254:1170-1177, (1979); and Hook et al., *FASEB J.* 8:1269-1278, (1994)). Thus, a condition for cleavage of the APP substrate includes an incubation
20 solution having a pH of about 7.0 or less. But secretases in vesicles are released by cells into the
25 extracellular environment, which can have a neutral or basic pH. Thus, vesicles can contain secretases that function at the neutral or basic pH of the extracellular
30 environment and, as such, that pH can also be an appropriate condition. The pH of the incubation solution
20 can be adjusted using known buffers (see Voet et al., *Biochemistry*, John Wiley and Sons, Pub., pp. 35-39, (1990)). Such buffers include, for example, citric acid,
35 sodium phosphate, MES, HEPES and Tris-HCl buffers. The pH of the incubation solution can be determined using
25 known methods such as, pH color indicators in liquid or paper formats, or pH meters. Examples III, IV, VIII, and IX show that the pH of the incubation solution can
40 affect the activity of secretases.

45 Other conditions that affect the cleavage
30 include the incubation temperature and incubation time. Proteolytic activity is a function of temperature with
50 excessively low or high temperatures resulting in no detectable activity. An incubation temperature thus is

any temperature which allows detection of a cleaved APP substrate. Usually an incubation temperature of about 30° to 45°C, with a typical temperature of about 35° to 40°C, and often a temperature of about 37°C is used. Although not required, a constant temperature during the incubation time is preferred and can be achieved using an incubator, water bath or other known means. An insufficient or excessive incubation time results in too little production or too much degradation of the product to be detected. The incubation time for cleavage of an APP substrate is that amount of time which allows cleavage of the APP substrate to be detected. A preferred incubation time allows the cleavage of an APP substrate to go to completion, for example, in about 2 to 8 hours.

The proteolytic activity of a secretase is determined by the cleavage of an APP substrate. An "APP substrate" as used herein is a compound having a stereochemical structure that is the same as, or a mimic of, an amino acid sequence in an APP protein, an A β peptide, an α -APP fragment or a 10 kDa fragment recognized by a secretase. Thus, an APP substrate for detecting a β - or γ -secretase includes, for example, the APP₆₉₅, APP₇₁₁, APP₇₅₁, and APP₇₇₁ proteins and an APP substrate for detecting an α -secretase includes, for example, those proteins and the A β peptides. As discussed above, such proteins, peptides and fragments have been isolated and characterized (Kang et al., *Nature* 325:733-736, (1987); Kitaguchi et al., *Nature* 331:530-532, (1988); Ponte et al., *Nature* 331:525-527, (1988); Tanzi et al., *Nature* 331, 528-530, (1988); Tanzi et al., *Science* 235:880-884, (1987), Glenner et al., *Biochem. Biophys. Res. Commun.* 120, 885-890, (1984); Masters et al., *Proc. Natl. Acad. Sci. USA* 82: 4245-4249,

5 (1985); Selkoe et al., *J. Neurochem.* 146: 1820-1834,
(1986); Selkoe, *J. Biol. Chem.* 271:18295-18298, (1996);
Mann et al., *Amer. J. Pathology* 148: 1257-66, (1996);
10 Masters et al., *Proc. Natl. Acad. Sci. USA* 82: 4245-4249,
5 (1985); Selkoe et al., *J. Neurochem.* 146: 1820-1834,
(1986); Selkoe, *J. Biol. Chem.* 271:18295-18298, (1996);
and Mann et al., *Amer. J. Pathology* 148: 1257-66,
15 (1996)).

Such APP substrates can be produced by various
10 methods known in the art (Knops et al., *J. Biol. Chem.*
266:7285-7290, (1991); Hines et al., *Cell. Molec. Biol.*
Res. 40:273-284, (1994)). For example, the APP proteins
can be made using recombinant technology and cloning the
25 cDNA that encodes the proteins into a suitable expression
15 system. An APP protein cDNA can be obtained, for
example, by screening a human brain cDNA library with a
DNA probe consisting of an oligonucleotide complementary
30 to the APP protein cDNA, a PCR-generated DNA fragment of
the APP protein cDNA, or a DNA fragment of the APP
20 protein cDNA from an expressed sequence tagged (EST)
database. Expression systems to produce APP proteins
35 include, for example, *E. coli.*, baculovirus-infected
insect cells, yeast cells, and mammalian cells.
Alternatively, such proteins can be produced using in
40 vitro methods, which transcribe and translate the RNA
that encodes these proteins to produce the proteins. An
APP so produced can be purified using methods such as
described herein or otherwise known in the art.

45 An APP substrate is also an APP
30 substrate-fusion substrate, in which a protein or peptide
is attached to an APP substrate for the purpose of
facilitating the isolation of the APP substrate.
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Proteins or polypeptides that facilitate purification include, for example, maltose-binding protein and multi-histidine polypeptides attached to the amino or carboxyl terminal end of the APP substrate. Thus, an example of an APP-fusion substrate is a multi-histidine polypeptide attached to the carboxyl terminus of an APP₆₉₅, APP₁₁₄, APP₇₅, or APP₇₇₁ protein. Such APP-fusion substrates can be produced using known methods such as by expression of the cDNA that encodes the APP-fusion substrate in a suitable expression system or in vitro translation of the encoding RNA. The APP-fusion substrates so produced can be purified by affinity binding to a column, such as by amylose, nickel or anti-APP antibody column chromatography.

Peptides are also known to function as protease substrates (see Sarath et al., *Protease assay methods*, In: *Proteolytic Enzymes, A Practical Approach*, R.J. Beynon and J.S. Bond, Eds., Oxford University Press, Pub., Chapter 3, pp 25-55, (1989). Often such a peptide substrate will contain the amino acids at a scissile bond in a precursor protein (see Benyon et al., *The Schechter and Berger Nomenclature for Protease Substrates*, In: *Proteolytic Enzymes, A Practical Approach*, R.J. Beynon and J.S. Bond, Eds., Oxford University Press, Pub., especially, Appendix 1, pp 231, (1989); and Barrett, *An Introduction to the Proteinases*, In: *Proteinase Inhibitors*, A.J. Barrett and G. Salvesen, Eds., Elsevier, Pub., Chapter 1, pp. 3-18, (1986)). A scissile bond is the peptide bond cleaved by a protease in a precursor protein. The amino acid on the amino terminal side of the scissile bond is often called the P1 amino acid and that on the carboxyl terminal side the P1' amino acid.

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A protease that cleaves a scissile bond binds the P1 and P1' amino acids. For some proteases, the P1 amino acid is the primary determinant for protease binding to the precursor protein. For example, the protease trypsin is known to have a marked preference for binding basic P1 amino acids. Peptide substrates often contain the amino acids attached to the amino terminal side of a P1 amino acid because those amino acids can influence the determinant effect of the P1 amino acid.

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10 An APP substrate also includes a peptide having an amino acid sequence recognized by a secretase containing a P1 or P1' amino acid, or both, of a scissile bond in an APP protein and one or more of the amino acids in the APP protein adjacent to either the P1 or P1' amino acids or both. For example, as shown in Figure 1, a β -secretase scissile bond is between the P1 amino acid methionine (Met or M) and the P1' amino acid aspartic acid (Asp or A). A β -secretase recognition site thus includes, for example, a Met-Asp substrate.

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20 Often an APP substrate is a peptide containing the P1 and P1' amino acids of a scissile bond in an APP protein and the one or two amino acids in the APP protein attached to the amino terminal side of the P1 amino acid. For example, as shown in Figure 1, a lysine (Lys or K) is attached to the amino terminal side of the P1 amino acid of the β -secretase scissile bond and a valine (Val or V) is attached to the amino terminal side of the Lys. Thus, an APP substrate for the β -secretase includes the Lys-Met-Asp and Val-Lys-Met-Asp (SEQ. ID NO.:1) substrates.

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The APP substrate peptide containing the P1 and P1' amino acids of a scissile bond in an APP protein can

5 be determined for the γ -secretase and the α -secretase in
the same manner. For example, as shown in Figure 1, the
10 γ -secretase scissile bond of the $A\beta_{1-40}$ peptide has a Val
P1 amino acid, an isoleucine (Ile or I) P1' amino acid, a
5 second Val attached to the amino terminal side of the P1
amino acid and a glycine (Gly or G) attached to the amino
terminal side of the second Val. As such, the
15 γ -secretase recognition site for the $A\beta_{1-40}$ peptide
includes, for example, the Val-Ile, Val-Val-Ile and
10 Gly-Val-Val-Ile (SEQ ID NO.:2) substrates. The
 γ -secretase recognition site for the $A\beta_{1-42}$ peptide thus
20 includes, for example, the Ala-Thr, Ile-Ala-Thr and
Val-Ile-Ala-Thr (SEQ ID NO.:3) substrates and that the
 γ -secretase recognition site for the $A\beta_{1-43}$ peptide
15 includes, for example, the Thr-Val, Ala-Thr-Val, and
Ile-Ala-Thr-Val (SEQ ID NO.:4) sequences. Similarly, the
 α -secretase recognition site can be determined from the
amino acids in the APP protein surrounding the
30 α -secretase scissile bond.

20 Proteases are known to have endoprotease,
aminopeptidase, or carboxypeptidase activity, or a
combination of these activities (see Sarath et al.,
35 *ibid.*). A protease having endoprotease activity cleaves
the peptide bond between two adjacent amino acids,
25 neither of which is a terminal amino acid, or, as
discussed below, between a non-terminal amino acid and a
terminal blocking group. A protease having
aminopeptidase activity only cleaves the peptide bond
between the amino terminal amino acid and its adjacent
40 amino acid. A protease having carboxypeptidase activity
only cleaves the peptide bond between the carboxyl
terminal amino acid and its adjacent amino acid.

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Secretases of the invention also can have endoprotease, aminopeptidase, or carboxypeptidase activity, or a combination of these activities. For example, an A β peptide can be cleaved from an APP protein directly by endoprotease cleavage of the scissile bonds at both ends of the A β peptide. But an A β peptide also can be produced by an endoprotease cleavage of a scissile bond distal to the terminal amino acids of the A β peptide followed by aminopeptidase or carboxypeptidase cleavage of the amino acids flanking the terminal amino acids of the A β peptide.

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An APP substrate often contains one or more amino terminal or carboxyl terminal blocking groups, which prevent aminopeptidase or carboxypeptidase cleavage, respectively (see Sarath et al., *ibid.*). But an amino terminal blocking group does not prevent carboxypeptidase and, conversely, a carboxyl terminal blocking group does not prevent aminopeptidase cleavage. As such, an APP substrate can often contain both an amino terminal and carboxy terminal blocking group to prevent both aminopeptidase and carboxylpeptidase cleavage. An APP substrate containing both blocking groups can only be cleaved, if at all, by a secretase having endoprotease activity.

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Blocking groups and methods of making substrates containing blocking groups are known in the art (see, for example, *Methods in Enzymology*, Vol. 244, "Proteolytic Enzymes," A.J. Barrett, Ed., Chapters 46, 47, and 48, (1994); and Green and Wuts, *Protective Groups in Organic Synthesis*, John Wiley and Sons, Pub., (1991) which are herein incorporated by reference). Amino terminal blocking groups include, for example, acyl (Ac), benzoyl (Bz), succinyl (Suc), carbobenzoxy (Z),

5 *p*-bromocarbobenzoxy, *p*-chlorocarbobenzoxy,
6 *p*-methoxycarbobenzoxy, *p*-methoxyphenylazocarbobenzoxy,
7 *p*-nitrocarbobenzoxy, *p*-phenylazocarbobenzoxy,
8 tert-butoxycarbonyl (Boc), benzoyl and the like. Carboxyl
9 blocking groups include, for example,
10 aminomethylcoumarinamide (MCA), the diazomethanes, the
11 *p*-nitroanilide (pNA), pNA-Tosylate, 2-naphthylamine, the
12 acyloxymethanes, including the (benzoyloxy)methanes,
13 (alkyloxy)methanes, the N,O-diacyl hydroxamates,
14 including the N-aminoacyl-O-4-nitrobenzoyl hydroxamates,
15 esters, including methyl, ethyl and nitrophenyl esters,
16 chloromethylketone and the like.

17 Although endoproteases do not cleave terminal
18 amino acids, endoproteases can cleave a carboxyl terminal
19 blocking group attached via a peptide bond to the
20 carboxyl terminal amino acid of a peptide containing two
21 or more amino acids (see Sarath et al., *ibid.*). If the
22 carboxyl terminal amino acid is the P1 amino acid of a
23 scissile bond in a precursor protein, the carboxyl
24 terminal blocking group mimics the P1' amino acid in that
25 scissile bond. Moreover, endoprotease cleavage of the
26 carboxyl terminal blocking group mimics the cleavage of
27 the corresponding scissile bond in the precursor protein.
28 Such carboxyl terminal blocking groups include, for
29 example, MCA, pNA, and pNA-Tosylate. An APP substrate
30 which contains such a carboxyl terminal blocking group
31 and an amino terminal blocking group can only be cleaved,
32 if at all, by an endoprotease.

33 An APP substrate includes a secretase
34 recognition site that contains a P1 amino acid of a
35 scissile bond in an APP protein and a carboxyl terminal
36 blocking group which replaces the P1' amino acid in that
37 scissile bond. The APP substrate also contains one or
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5 more of the amino acids in the APP protein attached to
the amino terminal side of the P1 amino acid. Such an
APP substrate will bind a secretase which binds the
10 corresponding scissile bond in the APP protein because
5 the substrate contains the P1 amino acid, the primary
determinant for that binding. For example, a β -secretase
recognition site containing such a carboxyl terminal
15 blocking group includes, for example, the Val-Lys-Met-MCA
substrate in which the MCA group replaces the Asp P1'
10 amino acid of the β -secretase scissile bond.
Endoprotease cleavage of the Met-MCA peptide bond in that
20 substrate is equivalent to endoprotease cleavage of the
scissile bond Met-Asp of the β -secretase recognition site
in the APP protein. Similarly a γ -secretase recognition
25 site for the $A\beta_{1-40}$ peptide includes, for example, the
Gly-Val-Val-pNA substrate in which the pNA group replaces
the Ile P1' amino acid of the corresponding γ -secretase
recognition site and endoprotease cleavage of the pNA
30 group is equivalent to endoprotease cleavage of the
20 corresponding scissile bond in the APP protein. Similar
substrates are envisioned for the γ -secretase recognition
site for the $A\beta_{1-42}$, and $A\beta_{1-43}$ peptides and the α -secretase
35 recognition site.

The APP substrate as discussed in the paragraph
25 above can also contain an amino terminal blocking group.
Only those secretases having endoprotease activity will
40 cleave that APP substrate and the endoprotease cleavage
of the substrate will mimic that which occurs in the APP
protein. Examples of such APP substrates include, but
45 are not limited to, Z*Lys-Met-MCA, Z*Val-Lys-Met-MCA,
30 Z*Val-Val-MCA, Z*Gly-Val-Val-MCA, Z*Ile-Ala-MCA,
Z*Val-Ile-Ala-MCA, Z*Ala-Thr-MCA, and Z*Ile-Ala-Thr-MCA
substrates. In these examples, Z represents the amino
50 terminal blocking group carbobenzoxy and the star (*)

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indicates a non-peptide bond between the Z and the adjacent amino acid. The MCA represents the carboxyl terminal blocking group aminomethylcoumarinamide and the dashes (-) represent peptide bonds between the MCA and the adjacent amino acid or between adjacent amino acids.

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Secretases having aminopeptidase activity can be assayed for using an APP substrate that contains an amino acid of a secretase recognition site and a carboxyl terminal blocking group. Examples of such APP substrates include Met-MCA and Lys-MCA substrates from the β -secretase recognition site. However, if such substrates contain only one amino acid, the substrate cannot be cleaved by an endoprotease because the only amino acid is an amino terminal amino acid. The Met-MCA and Lys-MCA substrates were used to identify β -secretase aminopeptidase secretase activities (see Example IV).

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An APP substrate often contains one or more labels that facilitate detection of the substrate or the APP derived product. A label can be an atom or a chemical moiety. Substrates containing a label can be made by methods known in the art. For example, radioactive atoms such as ^3H or ^{32}P can be attached to an APP substrate to detect an APP derived product. Also, heavy atoms or atom clusters such as, gold clusters can be attached. Moreover, fluorescent molecules such as, fluorescein, rhodamine, or green fluorescent protein, can be attached. A label can have more than one function. For example, the MCA is a carboxyl blocking group that is not fluorescent when bound in an APP substrate, is an APP derived product when cleaved by an endoprotease from a substrate, and is a label because, when MCA is cleaved from the substrate, it becomes fluorescent aminomethylcoumarinamide (AMC or free MCA) which is

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5 detectable (Azaryan and Hook, *Arch. Biochem. Biophys.*
314:171-177, (1994); and Azaryan et al., *J. Biol. Chem.*
270:8201-8208, which are incorporated herein by
10 reference).

5 Cleavage of an APP substrate can be detected by
the presence of an APP-derived product. The term "APP
15 derived product" refers to a protein, polypeptide,
peptide or chemical moiety produced by proteolytic
cleavage of an APP substrate. An APP derived product
20 includes, for example, an A β peptide, an α -APP fragment,
a 10 kDa fragment, and AMC. A chemical moiety is the
blocking group or label discussed above.

25 An APP derived product or an APP protein can be
qualitatively or quantitatively detected using various
15 methods. For example, these products or proteins can be
detected by an immunoassay using antibodies such as
monoclonal or polyclonal antibodies against the A β ₁₋₄₀
30 peptide, A β ₁₋₄₂ peptide, A β ₁₋₄₃ peptide, the amino terminal
or the carboxyl terminal regions of the APP proteins and
20 the APP proteins. Such antibodies are commercially
available, for example, from PENINSULA LABORATORIES,
35 Belmont, CA; CALBIOCHEM, San Diego, CA; QCB, Hopkinton,
MA; or IMMUNODYNAMICS, La Jolla, CA.

40 SDS-PAGE electrophoresis and western blots can
25 also be used to detect an APP derived product and an APP
protein (see Example XII). Other methods include
detecting a label on or from the APP derived product or
45 APP protein such as a radioactive or fluorescent label.
Microsequencing, amino acid composition analysis, or mass
30 spectrometry analysis can also be used (see Example XV).
Chromatography separation methods based on physical
50 parameters such as molecular weight, charge, or

5 hydrophobicity can be used. Preferred chromatography
methods include high pressure liquid chromatography
(HPLC) and automated liquid chromatography (FPLC,
10 PHARMACIA, Piscataway, NJ). Spectrophotometric
5 detection methods such as UV absorbance at 280 nm or
210-215 nm, can also be used. Known light or electron
microscopic methods as well as fluorescent activated cell
15 sorter methods also can be used to detect APP derived
products and APP proteins. The quantitative fluorescence
10 analysis using a fluorometer was used to detect the
fluorescent AMC product produced by β -secretase cleavage
20 of the Z*Val-Lys-Met-MCA, Met-MCA, and Lys-MCA (see
Examples III, IV, VIII, and IX).

25 Figure 2 shows the endoprotease cleavages that
15 can occur in an APP substrate containing a β -secretase
recognition site and amino and carboxyl terminal blocking
groups and how such cleavages can be detected. In that
figure, the three endoprotease cleavages of the APP
30 substrate Z*Val-Lys-Met-MCA are shown (#1, #2, and #3).
20 The Met-MCA bond (#3) mimics the scissile bond between
the P1 and P1' amino acids Met and Asp in the APP protein
at the amino terminal end of the A β peptide.
35 Endoprotease cleavage of the Met-MCA bond in the
substrate is equivalent to endoprotease cleavage of the
25 APP protein. That cleavage in the APP protein would
40 produce directly the amino terminal end of the A β
peptide. That cleavage can be detected by the
characteristic fluorescence produced by AMC (free NCA).

45 Endoprotease cleavage of the Lys-Met bond (#2)
30 and the Val-Lys bond (#3) in the Z*Val-Lys-Met-MCA
substrate produces a Met-MCA and Lys-Met-MCA peptide,
respectively. The corresponding endoprotease cleavages
50 in the APP proteins would be distal to the amino terminal

5 end of the A β peptide. However, such distal endoprotease
cleavages can occur *in vivo* because, as discussed above,
such cleavages followed by aminopeptidase cleavage of the
10 flanking amino acids can produce the amino terminal end
5 of the A β peptide.

15 The Met-MCA and Lys-Met-MCA peptides are not
fluorescent, but contain free amino terminal amino acids,
which an aminopeptidase can cleave to liberate AMC. To
insure that the endoprotease cleavages of the Lys-Met and
10 the Val-Lys bonds are detected, an aminopeptidase can be
20 added to an incubation solution to liberate AMC from the
Met-MCA and Lys-Met-MCA peptides. Known aminopeptidases
include, for example, aminopeptidase M and methionine
aminopeptidase (*Mammalian Proteases, a Glossary and*
25 *Bibliography*, J.K. McDonald and A.J. Barrett, Ed.,
15 Academic Press, Pub., p. 23-99, (1986)). In this manner,
all the endoprotease cleavages of the Z*Val-Lys-Met-MCA
substrate can be detected.
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Such methods were used to identify endoprotease
20 activity of one or more β -secretases in substantially
35 purified vesicles (see Examples III, VIII, and IX). In
particular, a secretase in substantially purified
vesicles was shown to cleave the Z*Val-Lys-Met-MCA
substrate at a pH of about 4.0 to about 5.5 using these
40 25 methods.

METHODS OF ISOLATING A SECRETASE

45 The present invention also is directed to a
method of isolating a secretase using the assay described
above to determine the proteolytic activity of a
30 secretase and isolating that secretase from
50 substantially purified vesicles. Generally, the

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isolation is done by assaying the activity of the secretase after each step in the isolation. If necessary, the activity can be preserved during the isolation procedure using methods such as those described above, including, for example isolating the secretase at a low temperature (e.g. 4°C), or in the presence of one or more of the above-described reducing or stabilizing agents.

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The secretase is isolated based on its physical properties. For example, a secretase can be isolated based on its molecular weight and size using gel filtration chromatography such as, Sephacryl S200, Sephadex G150, Superose 6 or 12, and Superdex 75 or 200 resin chromatography. A secretase can also be isolated based on its charge using ion-exchange chromatography such as DEAE-Sephadex, CM Sephadex, MonoQ, MonoS and MonoP resin chromatography. In addition, a secretase can be isolated based on its water solubility using hydrophobicity chromatography such as phenyl Sepharose, butyl Sepharose and octyl Sepharose resin chromatography. Interactions between the secretase and hydroxyapatite can also be used for isolation using, for example, macro-prep hydroxyapatite, and Bio-Gel HT hydroxyapatite resins.

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A secretase can also be isolated based on specific biochemical properties of the secretase using affinity chromatography. For example, the secretase can be isolated using APP substrate affinity chromatography under conditions in which the secretase binds the APP substrate but does not cleave it. Glycosylated secretases can be isolated using lectin affinity chromatography such as, concanavalin A-Sepharose, lentil lectin Sepharose, wheat germ lectin Sepharose resin chromatography. The proteolytic activity of sulfhydryl

5 groups such as those on cysteine amino acids can be used
to isolate the secretases using thiol-propyl
10 chromatography. Finally, the affinity of the secretases
for specific dyes can be used for separation such as,
5 blue-Sepharose resin chromatography. Other affinity
chromatography methods include arginine-Sepharose,
benzamidine Sepharose, glutathione Sepharose,
15 lysine-Sepharose and chelating Sepharose resin
chromatography. The secretases can also be isolated by
10 non-chromatographic fractionation methods using, for
example, native gel electrophoresis, analytical
20 ultracentrifugation and differential ammonium sulfate
precipitation methods (see Example XII).

25 Using such methods, alone or in combination, a
secretase of the invention can be isolated. The term
"isolated" when used in reference to a secretase means
that the secretase is relatively free of other proteins,
30 amino acids, lipids and other biological materials.
normally associated with a cell. Generally, an isolated
20 secretase constitutes at least about 50%, and usually
about 70% to 80%, and often about 90 to 95% or more of
35 the biological material in a sample. A secretase often is
isolated such that it is free of other substances that
affect the cleavage of an APP substrate, such as an
25 inhibitor or activator protein. The extent to which the
secretases are isolated using such methods can be
40 determined by known protein assays. For example, the
amount of protein in the resulting chromatographic
fractionation can be quantitated using the Lowry method
45 and the specific activity can be used to quantitate the
isolation (see Example XIII). Alternatively, SDS-PAGE or
two-dimensional gel electrophoresis and mass spectroscopy
50 methods can be used.

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After initial isolation of a secretase, antibodies specific to the secretase can be produced and secretases isolated using immunoaffinity chromatography. Such antibodies can be produced using known immunological methods including, for example, monoclonal antibody and polyclonal antibody production methods (see Haylow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, (1988)).

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The amino acid sequence of the secretase also can be determined after isolation of the secretase. For example, the amino acid sequence of the secretase can be determined using peptide microsequencing methods known in the art (see *"Current Protocols in Protein Science,"* Vol. 1 and 2, Coligan et al., Ed., (1997), John Wiley and Sons). Alternatively, the partial amino acid sequence can be determined from fragments of the secretase using mass spectrometry and Edman microsequencing methods (*"Current Protocols in Protein Science,"* Vol. 1 and 2, Coligan et al., Ed., (1997), John Wiley and Sons). For example, the secretase can be isolated using an SDS-PAGE gel and stained with coomassie blue in the gel. The secretase in the gel can be subjected to in-gel tryptic digestion and the amount of protein determined by amino acid analysis. Tryptic peptide fragments can be separated by HPLC, and the amino acid sequence of each fragment determined by Edman microsequencing and mass spectrometry methods. The amino acid sequence of the secretase can be determined from the amino acid sequences of the peptide fragments using computer analysis of known amino acid sequences.

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Based on the partial amino acid sequence of a secretase, the cDNA of the secretase can be cloned (see, for example, *Molecular Cloning, a Laboratory Manual*, Vol.

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1, 2, and 3, Sambrook et al., Ed., Cold Spring Harbor Laboratory Press, Pub., (1989); and *Current Protocols in Molecular Biology*, Vol. 1, 2, and 3, Ausubel et al., Ed., Wiley Interscience, Pub., (1997)). Briefly, partial, cloned secretase cDNAs are obtained by reverse transcription-polymerase chain reaction methods (RT-PCR) using oligonucleotides complementary to the partial amino acid secretase sequences. The complementary oligonucleotides synthetically synthesized can contain either degenerate codons, including inosine, or be optimized for mammalian cell use. The PCR-generated DNA fragment is analyzed for nucleic acid sequences and restriction enzyme sequences, and overlapping sequences among the different PCR-generated DNA fragments are determined. Northern blot or RT-PCR analysis using the PCR-generated cDNAs, or complementary oligonucleotides, so produced are used to determine tissues that produce mRNAs encoding the secretase. A cDNA library from such tissues is constructed and screened using the PCR-generated secretase cDNA or the complementary oligonucleotides. From such screened cDNA libraries, the cDNA sequence encoding the full-length amino acid sequence of the secretase is determined.

The cDNA of a secretase can also be obtained by generating antibodies against the partial amino acid sequences, screening cDNA expression libraries with an anti-secretase antibody, and analyzing the nucleic acid sequences of such clones. The amino acid sequence of the secretase can be deduced from the secretase cDNA sequence. The full-length cDNA can be cloned in an expression system such as in *E. coli*, Sf9 insect cells, yeast, or mammalian cell lines, and the activity of the expressed secretase determined to confirm that the cDNA encodes a functional secretase.

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Another method of obtaining the cDNA of a secretase is to clone the secretase in a genetic screen for isolating the secretase cDNA using the bacteriophage 1 regulatory circuit, where the viral repressor is
5 specifically cleaved to initiate the lytic phase of bacteriophage to allow detection and isolation of plaques containing the secretase cDNA(s) (Sices and Kristie, *Proc. Natl. Acad. Sci. USA* 95:2828-2833, (1988)).

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The gene(s) encoding a secretase can be isolated by screening a genomic library with the cDNAs encoding the partial or full length secretase, or with the oligonucleotides that are complementary to a sequence encoding a determined secretase amino acid sequence. The nucleic acid sequence of the secretase genomic DNA is
15 determined, and the exon/intron structure of the secretase gene is determined by comparing the DNA sequence of the gene to the nucleic acid sequence of the secretase cDNA.

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Once the cDNA encoding a partial or full-length endogenous secretase is obtained from one animal species, that cDNA can be used to obtain endogenous secretases from another animal species using known methods (*Molecular Cloning, a Laboratory Manual*, *ibid.*; and *Current Protocols in Molecular Biology*, *ibid.*). For
25 example, the cDNA encoding the partial bovine secretase can be used to obtain cDNAs encoding human secretases. Briefly, a partial or full-length bovine cDNA, or a labeled complementary oligonucleotide, is used to isolate the human secretase cDNA by screening human cDNA
30 libraries constructed from tissues that contain secretase mRNA, determined by northern blot or RT-PCR analyses. Alternatively, the human secretase cDNA can be obtained by searching the expressed sequence tag database (EST)

5 for human cDNA sequences similar to the bovine secretase
cDNA. DNA sequencing of the resulting secretase clones
10 can be performed to determine the nucleic acid sequence
encoding the human secretase and the corresponding amino
5 acid sequence can be deduced. The cDNA encoding the
human secretase can be cloned in and expressed by a
suitable expression vector and the activity of the
15 expressed secretase can be determined. The genes
encoding the human secretase can be cloned as described
10 herein.

20 The nucleic acid sequence of a secretase can
also be used to produce the secretase using known
recombinant methods (*Molecular Cloning, a Laboratory*
25 *Manual, ibid.*; and *Current Protocols in Molecular*
15 *Biology, ibid.*). The cDNA encoding the secretase can be
inserted into an appropriate expression vector and the
expression vector introduced into an appropriate host as
described herein. Expression of the secretase by the
30 host is stimulated by expression of a vector promotor.

20 METHODS OF SCREENING FOR AGENTS THAT AFFECT THE
35 PROTEOLYTIC ACTIVITY OF A SECRETASE

40 Another aspect of the invention is a method of
selecting an agent that alters the cleavage of an APP
substrate by a secretase. Such agents, particularly
25 those that decrease the cleavage by the β -secretase and
 γ -secretases or that increase the cleavage by the
 α -secretase, are useful for developing drugs that prevent
45 or treat AD. Agents having divergent chemical structures
can be assayed using such methods including, for example,
30 small organic molecules that optionally contain
heteroatoms or metals, amino acids, peptides,
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5 polypeptides, proteins, peptidomimetics, nucleic acids,
carbohydrates, glycoproteins, lipids, and lipoproteins.

10 The method is based on comparing the APP
substrate cleavage, or the APP protein, or APP derived
5 product production that occurs with and without an agent.
This is achieved by determining the APP substrate
15 cleavage or the APP protein or the APP derived product
produced in a first incubation or culture solution
lacking the agent and comparing that result with that
20 which occurs in a second incubation or culture solution
containing the agent. The first and second incubation or
culture solutions can be different solutions or the same
solution to which the agent is added or removed. The APP
25 substrate cleavage, the APP protein, and the APP derived
15 product can be assayed using the methods described
herein. The concentration of the agent can vary due to
parameters known in the art such as the hydrophobicity,
30 charge, size and potency of the agent, but typically is
about a 10^{-9} to 10^{-3} M.

20 Agents are selected that alter the cleavage of
an APP substrate or production of an APP protein or an
35 APP derived product. The cleavage or production is
altered if the agent causes a significant change in the
cleavage or production relative to that which occurs
40 without the agent. A significant change can be
25 determined using a variety of qualitative or quantitative
methods, such as, for example, by a visual or statistical
analysis of the comparison data. For example, the mean
45 amounts of an APP derived product obtained with and
30 without the agent can be analyzed using a two-sided
Student's t-test and a $p \geq 0.02$ or greater, and
preferably a $p \geq 0.05$, in that test can be indicative of
50 a significant difference.

5 Often agents are screened using substantially
pure vesicles as the source of the secretase. But
substantially pure vesicles can contain, in addition to
10 secretases, other substances that affect the cleavage of
an APP substrate, such as the presenilin 1 protein.
5 Thus, a screen using such vesicles selects for agents
that directly or indirectly alter the cleavage. An agent
15 can directly affect the cleavage by, for example,
inhibiting the binding of an APP substrate to a
secretase. But an agent can also indirectly alter the
10 cleavage by affecting an inhibitor or activator substance
which in turn affects the activity of the secretase.
20 For example, proteases may be present in the vesicle that
produce the secretase from a precursor protein or that
25 degrade the secretase. An agent thus can indirectly
affect the secretase activity by affecting the proteases
which produce or degrade the secretase. Often
30 permeabilized chromaffin vesicles and an APP protein, A β
peptide, Z*Val-Lys-Met-MCA, Z*Gly-Val-Val-MCA,
20 Z*Val-Ile-Ala-MCA, or Z*Ile-Ala-Thr-MCA substrate are
used in the assay.

35 An isolated secretase, obtained as described
above, can also be used to select for agents that affect
the activity of the secretase. Using an isolated
25 secretase free of other substances that affect the
cleavage of an APP substrate, agents can be selected that
40 directly affect cleavage of the APP substrate. The
effect of an agent on such an isolated secretase and on
substantially purified vesicles can be compared to
45 determine the direct and indirect effects of the agent.
30 Moreover, that comparison can be used to determine if
the vesicles contain inhibitors or activators of the
secretase removed during isolation of the secretase.
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5 The protease class to which an isolated
secretase belongs can be determined using agents known to
selectively inhibit different classes of proteases. For
10 example, E-64c, cystatin, and p-mercuribenzoate inhibit
5 cysteine proteases; phenylmethylsulfonyl fluoride (PMSF),
soybean trypsin inhibitor, and α_1 -antitrypsin inhibit
serine proteases; ethylenediaminetetraacetic acid (EDTA)
15 and 1,10-O-phenanthroline inhibit metalloproteases; and
pepstatin A inhibits aspartyl proteases. (See Examples
10 XI and XIV).

20 In another method, a cell containing vesicles
having the proteolytic activity of a secretase is used to
select for an agent. Cells containing such vesicles can
be identified using the methods described above to
25 determine the proteolytic activity of a secretase in the
vesicles. The cells are cultured in a first culture
solution without the agent and in a second culture
solution with the agent and the production of an APP
30 protein or an APP derived product by the cell, especially
20 an A β peptide, α -APP fragment or 10 kDa fragment, in the
first and second culture solution compared.

35 A problem with using transformed cell cultures
or cell lines to select agents is that the agents may be
ineffective *in vivo* because cells in culture can process
40 a protein in a manner unrelated to that which occurs in
25 *vivo*. Thus, agents that affect the processing of such
cells are ineffective because the processing that they
affect does not occur *in vivo*. The cell based method
45 provided in the present invention avoids this problem by
30 selecting cells determined to contain vesicles that have
the proteolytic activity of a secretase. As such, the
method insures that the cells process the APP protein in
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the cell organelle in which that processing occurs in vivo

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A cell used in this method can be obtained from a variety sources. For example, disassociated cells maintained in a primary culture can be used in the method. Such disassociated cells can be maintained in a primary culture using known methods (see, for example, Hook et al., *ibid.*; and Tezapsidis et al., *ibid.*). Disassociated cells have the advantage of retaining many of the functional characteristics that they have in the tissue that they are obtained from. But primary cultures of disassociated cells generally die after a period of time. Cell lines, transformed cells and cloned cells, on the other hand, have the advantage of being immortal. But such cells are known to often abnormally process proteins. As such, it is particularly important to use immortalized cells that are determined to contain vesicles in which the proteolytic activity of a secretase occurs so as to insure that the cells are processing the APP protein in the same manner as in vivo. Various cell transformation methods can be used to obtain such cells (see for example, Alarid et al. *Development*, 122(10):3319-29, (1996); and Schechter et al., *Neuroendocrinology*, 56(3):300-11, (1992), which are incorporated herein by reference). A chromaffin cell, either obtained by disassociation or by transformation, is often used in this method.

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In the cell based assay of the present invention, the agent is often present when the cells are producing an APP derived product because some agents are known to only affect a protease in a cell when the protease is producing a product. For example, agents are known to inhibit enkephalin production in chromaffin

5 cells only when the chromaffin cells are actively
producing enkephalin (Tezapsidis et al., *ibid.*). Various
10 methods can induce cells to produce proteolytically
processed peptides in vesicles. For example, proteolytic
5 processing can be induced by exocytosis. Exocytosis can
be induced by various means including, for example, by
15 increasing the extracellular potassium chloride
concentration or by binding nicotinic cholinergic
receptors on cells with nicotine. Proteolytic processing
10 of the A β peptides can also be induced by stimulating
protein kinase with phorbol esters (Koo, *Molec. Medicine*,
20 3:204-211, (1997); and LeBanc et al., *J. Neurosci.*,
18:2908-2913, (1998)).

25 For example, as shown in Example VII,
15 chromaffin cells can be induced to produce an A β peptide
by culturing the cells in potassium chloride (about 5 to
500 mM), nicotine (about 10^{-3} to 10^{-6} M), or phorbol ester
30 (about 10^{-3} to 10^{-6} M) for a sufficient amount of time to
stimulate production (about 1 to 72 hours for the
20 nicotine and potassium chloride and about 12 to 96 hours
for the phorbol ester). During active production of the
A β peptide by the cells, an agent is incubated with the
35 chromaffin cells under appropriate conditions and for an
appropriate amount of time (e.g. about 2 to 8 hours).
25 The cells can then be lysed and the production of an A β
peptide with and without the agent compared. To
40 facilitate that comparison, a protease inhibitor such as,
chymostatin, leupeptin, and soybean trypsin inhibitor
(STI), can be added when cells are lysed to prevent
45 30 non-specific digestion of the A β peptide by non-specific
proteases released by cell lysis.

50 The cell based assay can be used to select an
agent that affects cell expression. For example, the

5 expression of a nucleic acid that encodes a secretase can
be tested in such an assay. Inhibitors of gene
transcription, such as actinomycin D or an antisense
10 nucleic acid, or agents that modify protein transcription
5 factors that regulate gene expression, such as steroids,
also can be tested. The cell based assay can also be
used to select agents that affect protein processing,
15 including those affecting RNA splicing, RNA
polyadenylation, RNA editing, protein translation, signal
10 peptidase processing, protein folding including
chaperone-mediated folding, disulfide bond formation,
20 glycosylation, phosphorylation, covalent modification
including methylation, prenylation, and acylation, and
association with endogenous protein factors that modify
15 secretase activity.

Agents found to alter cleavage of an APP
substrate can be evaluated *in vivo* using transgenic AD
30 animal models. Transgenic animal models have been
developed in which the animals have brain amyloid plaques
20 containing A β peptides and, in some models, exhibit
cognitive deficits such as excessive memory loss.
35 Exemplary transgenic animals include mice that contain
the Indiana mutation of the human APP cDNA under the
control of the PDGF promoter (Johnson-Wood et al., *Proc.*
25 *Natl. Acad. Sci., USA*, 94:1550-1555, (1997)). These mice
40 express increased levels of brain A β peptides and amyloid
plaques and show cognitive deficits. Another exemplary
transgenic animal is a mouse strain containing the
Swedish mutation of the human APP-695 cDNA with the
45 hamster PrP promoter (Hsiao, *J. Neural Transmission*,
30 49:135-144, (1997)). These mice express increased levels
of brain A β peptides, have amyloid plaques and are memory
impaired.

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Agents can be administered to such animals using methods known in the art, particularly those methods that result in the agent traversing the blood brain barrier. For example, such agents can be administered by direct injection into the central nervous system or by administration with a minipump. Agents that naturally traverse the blood brain barrier can be systematically administered by intravenous, subcutaneous, or oral routes. Such agents can be administered in effective doses which for example can range from 0.001 to 10 mg/kg body weight. Agents can be administered prophylactically or therapeutically in single or multiple dose schedules.

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Agents can be assayed by histopathological examination of the brains from such transgenic animals. For example, quantitative, microscopic analysis of amyloid plaque formation can be used to determine the effect of the agent. Agents which reduce the size or frequency of amyloid plaques are preferred. In addition, agents can be assayed by measuring brain levels of $A\beta_{1-40}$, $A\beta_{1-42}$, or $A\beta_{1-43}$ by radioimmunoassay or ELISA. Agents that reduce $A\beta_{1-40}$, $A\beta_{1-42}$, or $A\beta_{1-43}$ levels are preferred. Agents also can be assayed for their effect on the cognitive behavior of such animals using known methods. For example, the memory capability of mice can be determined using the water maze test. Agents which enhance the memory capability are preferred.

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Agents that effectively reduce or inhibit $A\beta$ peptide production or amyloid plaque formation or increase memory in any of the methods described above can be used to treat or prevent AD. Persons identified as probable AD patients by known medical methods can be administered such agents. Also, people diagnosed as

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having a high probability of developing AD can be administered such agents. Patients are assessed for improvement in cognitive abilities. Upon autopsy, brain tissue is assessed for amyloid plaques and A β levels. Agents are administered by known methods such as those described above for the animal model.

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Agents that effectively reduce or inhibit A β peptide production or amyloid plaque formation or increase memory can also be used to enhance memory function of people, especially the elderly. People can be administered such agents and assayed for improved memory capability. Agents can be administered by known methods such as those described above for the in vivo assay.

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The following examples are intended to illustrate but not limit the present invention.

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EXAMPLE I

Isolation of Chromaffin Vesicles

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Chromaffin vesicles were isolated from fresh bovine adrenal medulla by discontinuous sucrose gradient centrifugation (Krieger et al., *Biochemistry*, 31, 4223-4231, (1992); Yasothornsrikul et al., *J. Neurochem.* 70, 153-163, (1998)). Briefly, fresh bovine adrenal glands were dissected to obtain the medulla region. These medulla from 40 glands were homogenized in 200-250 ml ice-cold 0.32 M sucrose, and the homogenate was centrifuged at 1,500 rpm in a GSA rotor (Sorvall centrifuge) for 20 minutes at 4° C.

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The resultant supernatant was collected and centrifuged at 8,800 rpm in a GSA rotor (Sorvall centrifuge) for 20 minutes at 4° C to obtain a pellet of

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5 chromaffin vesicles. The pellet of chromaffin vesicles
was washed three times in 0.32 M sucrose. Each wash
consisted of resuspending the pellet of chromaffin
10 vesicles with an equal volume (same volume as original
homogenate) of 0.32 M sucrose and centrifugation at 8,800
5 rpm in a GSA rotor to collect the vesicles as the pellet.

15 After washing, the chromaffin vesicles were
resuspended in 120 ml of 0.32 M sucrose and subjected to
discontinuous sucrose gradient centrifugation. For that
10 centrifugation, 10 ml of the washed chromaffin vesicle
suspension was layered on top of 25 ml of 1.6 M sucrose
20 in each of 12 centrifuge tubes. The 12 tubes of sucrose
gradient were centrifuged in a SW28 rotor at 25,000 rpm
for 120 minutes at 4° C. The pellets of isolated
25 chromaffin vesicles from 12 tubes were resuspended in 12
ml of 0.015 M KCl with a glass-glass homogenizer, and
stored at -70° C, prior to use. A chromaffin vesicle
30 lysate was prepared by freeze-thawing the isolated
chromaffin vesicles in the 15 mM KCl.

20 EXAMPLE II

35 Assay for Chromaffin Vesicles

The chromaffin vesicles in the Example I
preparation were assayed for the chromaffin vesicle
40 markers (Met)enkephalin, catecholamines, the lysosomal
marker acid phosphatase and total protein. Fractions
25 containing the highest amount of chromaffin vesicle
markers were identified as chromaffin vesicles. The
homogeneity of the chromaffin vesicles was approximately
45 99% as assayed by the proteolytic activity of the
chromaffin vesicle markers (Met)enkephalin and
30 catecholamines and the absence of the lysosomal marker
acid phosphatase. Electron microscopy showed that
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5 uniform, homogeneous, and intact chromaffin vesicles were
isolated. The chromaffin vesicles were purified
approximately 8-fold from the cell homogenate based on
10 the measurement of the picograms of (Met)enkephalin per
5 microgram of protein in the samples.

15 - EXAMPLE III

β -secretase Endoprotease Activity

The APP substrate, Z*Val-Lys-Met-MCA, was used
to identify a β -secretase based on endoprotease activity.
20 That substrate was commercially obtained and had a purity
of 99% or better as determined by the manufacturers
(PENINSULA LABORATORIES, Belmont, CA and PHOENIX
LABORATORIES, Mountain View, CA).

The β -secretase endoprotease activity was
15 identified by incubating the chromaffin vesicle lysate
(2-10 μ l of 10-20 mg protein/ml) with the
30 Z*Val-Lys-Met-MCA substrate (100 μ M final concentration)
and detecting AMC fluorescence. The chromaffin vesicle
lysate was prepared as described in Example I. The
35 endoprotease activity was determined as a function of pH
by varying the pH of the incubation solution between 3.0
to 8.0 in 0.5 pH increments. Citric acid, sodium
phosphate, and Tris-HCl buffers (100 mM final
40 concentration) were used to adjust the pH of the
25 incubation solutions between 3.0 to 5.5, 6.0 to 7.5, and
8.0, respectively. Duplicate samples at each pH
increment (100 μ l each) were distributed among 22 wells
45 in a covered microtiter well plate and incubated at 37° C
for 8 hours in a water bath.

30 As discussed above, endoprotease cleavage
50 between the Met-MCA bond in the Z*Val-Lys-Met-MCA

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5 substrate produces fluorescent AMC, but endoprotease
cleavage between the Lys-Met or Val-Lys bonds in that
10 substrate produces non-fluorescent Lys-Met-MCA and
Met-MCA peptides. To insure that the latter two
5 endoprotease cleavages were detected, aminopeptidase M
(20 µg/ml final concentration, BOEHRINGER MANNHEIM) was
added to each incubation solutions to produce fluorescent
15 AMC from the Lys-Met-MCA and Met-MCA peptides. Prior to
adding the aminopeptidase M, each incubation solution was
10 adjusted to a pH 8.3 using Tris-HCl because
aminopeptidase M functions at a basic pH. A second
20 incubation at 37° C for 1 hour in the water bath was
conducted to complete the aminopeptidase M reaction.

25 Upon termination of that second incubation, AMC
15 fluorescence was assayed using a fluorometer (IDEXX
fluorometer, FCA Fluorescence Concentration Analyzer,
cat. no. 10-105-2, BAXTER HEALTH CARE CORP., Mundelein,
30 IL) at excitation and emission wavelengths of 365 and 450
nm, respectively. Standard AMC concentrations were also
20 measured to quantitate relative fluorescence with the
molar amount (pmol) of AMC generated by the secretase.
35 The resulting AMC fluorescence reflects the endoprotease
activity in cleaving either the Met-MCA, Lys-Met. and
Val-Lys bonds in the Z*Val-Lys-Met-MCA substrate.

40 25 The AMC fluorescence was plotted as a function
of pH and is shown in Figure 3. Analysis of that plot
shows a principal β -secretase endoprotease activity
having a pH optimum of about 4.5-5.0. In addition, the
45 plot shows two lesser β -secretase endoprotease activities
30 having pH optimums of about pH 3.5 and 6.0-6.5.

EXAMPLE IV

 β -secretase Aminopeptidase Activity

The APP substrates, Met-MCA, and Lys-MCA, were used to identify a β -secretase based on aminopeptidase activity. Those substrates were commercially obtained and had a purity of 99% or greater as determined by the manufacturers (PENINSULA LABORATORIES, Belmont, CA and PHOENIX LABORATORIES, Mountain View, CA).

The β -secretase Met aminopeptidase activity was identified by incubating the chromaffin vesicle lysate (5 μ l of 10-15 mg/ml) with the Met-MCA substrate (100 μ M final concentration) and detecting the resulting AMC fluorescence. The chromaffin vesicle lysate was prepared as described in Example I. The aminopeptidase activity was determined as function of pH by varying the pH of the incubation solution between 3.0 to 8.0 in 0.5 pH increments. Citric acid, sodium phosphate, and Tris-HCl buffers (100 mM final concentration) were used to adjust the pH of the incubation solutions between 3.0 to 5.5, 6.0 to 7.5, and 8.0, respectively. Duplicate samples at each pH increment (100 μ l each) were distributed among 22 wells in a covered microtiter well plate and incubated at 37° C for 4 hours in a humidified incubator.

Similarly, the β -secretase Lys aminopeptidase activity was identified by incubating the chromaffin vesicle lysate (5 μ l of 10-15 mg/ml) with the Lys-MCA substrate (100 μ M final concentration) and detecting the resulting AMC fluorescence. The incubation was identical to that described for the Met aminopeptidase assay except that the incubation time was 2 hours long.

5 Upon termination of the incubations, AMC
fluorescence was assayed as described above. The
10 resulting AMC fluorescence indicating β -secretase Met and
Lys aminopeptidase activities was plotted as a function
5 of pH and is shown in Figures 4 and 5, respectively.

15 Analysis of Figure 4 shows a β -secretase Met
aminopeptidase activity having a pH optimum of about
5.5-6.5. Similarly, analysis of Figure 5 shows a
20 β -secretase Lys aminopeptidase activity having a pH
optimum of about 6.0-7.0.

EXAMPLE V

Identification of A β peptides

25 The chromaffin vesicle lysate was analyzed for
the proteolytic activity of A β peptides using
15 commercially available polyclonal and monoclonal
antibodies against the A β_{1-40} and A β_{1-42} (PENINSULA
30 LABORATORIES, Belmont, CA and QCB, Hopinton, MA,
respectively) in known radioimmunoassay (RIA) and ELISA
methods. The chromaffin vesicles contained A β_{1-40} at 0.051
35 20 pg/ug protein as determined by RIA and a detectable
amount of A β_{1-42} as determined by ELISA.

EXAMPLE VI

APP Protein Distribution in Chromaffin Cells

40 The distribution of APP protein in chromaffin
25 cells was determined using a monoclonal antibody directed
against the amino terminal region of the APP protein
45 (Anti-Alzheimer precursor protein A4, clone #22C11,
BOEHRINGER MANNHEIM, Indianapolis, IN) in established
immunofluorescent cytological methods. Fluorescent light
50 30 microscopic analysis of chromaffin cells stained by this

method showed that the APP protein was localized in the chromaffin vesicles and not in the cell nucleus.

EXAMPLE VII

A β -peptide Secretion by Chromaffin Cells

Primary chromaffin cell cultures containing approximately 2 million cells in each culture were produced using established methods (Hook et al., *ibid.*; and Tezapsidis et al., *ibid.*). Exocytosis of the contents of the vesicles in such cells was induced by exposing the cells to KCl (50 mM) or nicotine (10 μ M) for 15 minutes. The media was removed from the cells and the A β_{1-40} peptide in the media was determined using the RIA assay described in Example V. The KCl and nicotine exposure caused an approximately 350-fold and 550-fold increase in the concentration of A β_{1-40} peptide in the media, respectively, relative to that of a control media from a culture identically processed but which did not receive KCl or nicotine. The results show that chromaffin cells exocytosis results in the secretion of A β peptide.

EXAMPLE VIII

Effect of Reducing Agents on β -secretase Endoprotease Activity in Chromaffin Vesicles

The effect of the reducing agent dithiothreitol (DTT) on β -secretase endoprotease activity was determined using the assay described in Example III. Briefly, the lysed vesicles were incubated with the substrate Z*Val-Lys-Met-MCA in the presence or absence of 1 mM DTT and the resulting fluorescence plotted as a function of pH. Both with and without DTT, β -secretase endoprotease activity was detected and in both cases that activity had

5 pH optimum of about 4.0 to 6.0, which is consistent with
the intravesicular pH of chromaffin vesicles. But the
10 DTT resulted in a significant increase in the β -secretase
endoprotease activity, approximately 5-fold (see Figure
5 6). These results show that DTT, although not essential,
significantly increases β -secretase endoprotease
15 activity.

EXAMPLE IX

Effect of Aminopeptidase M on β -secretase Endoprotease.
10 Activity in Chromaffin Vesicles

15 The effect of the aminopeptidase M and the
basic pH buffer used in the β -secretase endoprotease
activity assay was determined. The assay was conducted
as described in Example VIII with DTT. Three assays were
20 conducted, one with aminopeptidase M in its basic pH
buffer, another with the basic pH buffer but not
aminopeptidase M, and a third without either the buffer
or the aminopeptidase M. Briefly, the chromaffin vesicle
lysate and the substrate Z-Val-Lys-Met-MCA were incubated
25 for 30 minutes at a specified pH and the resulting
fluorescence measured. The aminopeptidase M in the basic
pH buffer or that buffer alone (final concentration of 75
mM Tris-HCl pH 8.2) was added to the assay and incubated
an additional 60 minutes at 37°C. The resulting
30 fluorescence was plotted as a function of pH, which
showed that β -secretase endoprotease activity occurred in
the 3 assays (see Figure 7). The assay conducted with
aminopeptidase M and its basic pH buffer and that of the
control assay having just the basic pH buffer produced
45 approximately the same amount of fluorescence. This
result is consistent with that obtained in Example IV,
which showed that chromaffin vesicles contain an
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endogenous β -secretase methionine and lysine
aminopeptidase.

EXAMPLE X

 β -secretase Endoprotease Activity Obtained During
Isolation of Chromaffin Vesicles

The β -secretase endoprotease activity of
fractions obtained during the isolation procedure
described in Example I was determined at the pH optimum
of 5.5, with and without DTT using the assay described in
Example VII. The ratio of those activities (with/without
DTT) was calculated and the ratios obtained for the
fraction shown in Table I.

TABLE I

	FRACTION	RATIO
15	Adrenal Medulla Homogenate	4.7
30	Pellet from 1,500 rpm Centrifugation (nuclear fraction)	11.6
	Pellet from 1st 8,800 rpm Centrifugation (crude vesicle fraction)	3.2
35	20 Pellet from 2nd 8,800 rpm Centrifugation (washed vesicle fraction)	6.3
40	Pellet from 25,000 rpm Discontinuous Gradient Centrifugation (vesicle fraction)	11.0

The results show that β -secretase endoprotease
activity is enriched in the nuclear fraction and the
vesicle fraction. But, as described in Example VI, only
the chromaffin vesicles contain the APP protein, and thus
only in that fraction does the protease having
 β -secretase endoprotease activity also have access to the
APP protein substrate.

EXAMPLE XI

Protease Inhibitors of β -secretase Endoprotease Activity
in Chromaffin Vesicle Lysate

The effect of various protease inhibitors on β -secretase endoprotease activity in the lysate was determined at the pH optimum 5.5 in the assay described in Example IX containing aminopeptidase M. Protease inhibitors specific for various protease classes were used. The protease inhibitor was added to each assay at the start of the reaction at the appropriate concentration. The extent of inhibition was expressed as a percentage of the activity without the inhibitor (control). Triplicate assays varied by less than 10%. The results are shown in Table II.

TABLE II

PROTEASE CLASS	INHIBITOR (Concentration)	% CONTROL
Control	None	100
Cysteine	E64c (10 μ M)	0
Cysteine	pHMB (1 mM)	35
Serine	PMSF (100 μ M)	58
Serine	Chymostatin (10 μ M)	11
Aspartyl	Pepstatin A (10 μ M)	78
Metallo	EDTA (1 mM)	100
Metallo	EGTA (1 mM)	99
Nonspecific	Leupeptin (100 μ M)	0

The results show that the β -secretase endoprotease activity in the chromaffin vesicle lysate was completely inhibited by the cysteine protease class.

inhibitor E64c, and the nonspecific protease inhibitor leupeptin. The serine protease class inhibitor chymostatin and the cysteine protease inhibitor PHMB greatly inhibited activity. The apartyl protease class inhibitor pepstatin A slightly inhibited the activity and the metallo protease class inhibitors did not inhibit activity.

EXAMPLE XII

Isolation of β -Secretases from Chromaffin Vesicles

The chromaffin vesicle lysate was separated into 2 β -secretase endoprotease activity peaks (referred to as "Peak I" and "Peak II"). Peak I had about 3 times the total activity of Peak II and a different β -secretase endoprotease activity than did Peak II. The Peak I activity was very sensitive to the presence of aminopeptidase M in the assay whereas the Peak II activity was relatively insensitive to aminopeptidase M.

The Peak I center and range of activities had molecular weights of about 185 kDa, and about 180 to 200 kDa, respectively. Peak I was found to be a protease complex having a broad band of activity as determined by a native PAGE activity assay and 3 distinct activities corresponding to molecular weights of about 88, 81, and 61 kDa, in a non-reducing SDS-PAGE activity assay. Peak I was found to contain 3 proteins having molecular weights of about 88, 81, and 36 kDa, and 4 proteins having molecular weights of about 66, 60, 33, and 29 kDa, in a non-reducing and a reducing SDS-PAGE stained for proteins, respectively.

Peak II had a center and range of activities having molecular weights of about 65 kDa, and about 50 to

90 kDa, respectively. Peak II contained 2 proteins having different net electronegative charges and β -secretase endoprotease activity (referred to as "Peak II-A" and "Peak II-B").

ISOLATION OF PEAKS I AND II AND CHARACTERIZATION OF THE β -SECRETASE ENDOPEPTIDASE ACTIVITIES IN THOSE PEAKS

The procedure used to isolate Peaks I and II is diagrammed in Figure 8. The β -secretase endoprotease activity with and without aminopeptidase M was determined after each isolation step using the assay described in Example IX. Isolation steps that enriched that activity were selected. The total and specific activities after each isolation step are summarized in Example XIII. The β -secretase aminopeptidase activity was determined by the assay described in Example IV.

Preliminary experiments indicated that the β -secretase is present in chromaffin vesicles at a relatively low concentration. Thus, a very large number of bovine adrenal glands, approximately 2400, was used so that a sufficient amount the β -secretase could be obtained for analysis. Using the methods described in Example I, numerous chromaffin vesicle lysate preparations were made over a period of approximately 6 months and pooled.

A soluble extract and membrane pellet from the pooled lysate was made by ultracentrifugation at approximately 100,000 x g. The bulk of the activity was in the soluble extract and was aminopeptidase insensitive (see Krieger, T.K. and Hook, V.Y.H. *J. Biol. Chem.* 266, 8376-8383, (1991)). As such, it was concluded that the β -

5 secretase endoprotease activity was not bound to the
chromaffin vesicle membranes.

10 The soluble extract was separated by
concanavalin A-Sepharose resin chromatography (referred
5 to as "Con A") into bound and unbound fractions. The
Con-A bound fraction was subsequently eluted using
15 alpha-methylmannoside (referred to as the "eluted Con-A
bound fraction") and contained the bulk of the
beta-secretase endoprotease activity, but no beta-secretase
20 aminopeptidase activity. The unbound fraction (referred
to as the "Con-A unbound fraction"), in contrast,
contained beta-secretase methionine and lysine
aminopeptidase activity, but little beta-secretase
25 endoprotease activity. The Con-A step thus separated the
15 endogenous beta-secretase endoprotease and aminopeptidase
activities (see Krieger, T.K. and Hook, V.Y.H., *ibid.*).

30 The contents of the eluted Con-A bound fraction
were fractionated according to molecular size using a
Sephacryl S200 column (Krieger, T.K. and Hook, V.Y.H.
20 *ibid.*). That resulted in the Peak I and Peak II
beta-secretase endoprotease activities. The Peak I center
35 and range of activities corresponded to proteins having
molecular weights of approximately 185 kDa, and 180 to
200 kDa, respectively. The Peak II center and range of
40 25 activities corresponded to proteins having molecular
weights of approximately 65, and 50 to 90 kDa,
respectively (see Figure 9).

45 Peak I had more than 3 times the total
activity of Peak II, but the Peak I activity without
30 aminopeptidase M was only about 5% of that produced with
the aminopeptidase. Thus, Peak I was aminopeptidase
50 sensitive. Since Peak I alone did not produce much

5 fluorescence, the majority of the Peak I activity does
not cleave the Met-MCA bond in the Z*Val-Lys-Met-MCA
substrate because cleavage of that bond must occur to
10 produce fluorescent free MCA. But since the addition of
5 aminopeptidase M produced a significant amount of
fluorescence, the majority of the Peak I activity must
endoproteolytically cleave that substrate because that
15 cleavage must occur, for reasons discussed above, in
order for the aminopeptidase M to cleave the Met-MCA bond
10 and the Lys-Met bond and produce fluorescent free MCA.
The Peak I activity thus must cleave the Lys-Met or the
20 Val-Lys bond because those are the only other peptide
bonds in the substrate that can be cleaved. Moreover,
the fact that aminopeptidase M must be added to Peak I to
15 detect activity confirms that the Con-A isolation step
removed most of the endogenous aminopeptidases from the
eluted Con-A bound fraction.

30 As discussed above, the Met-MCA bond in the
20 Z*Val-Lys-Met-MCA substrate is a mimic of the β -secretase
scissile bond Met-Asp in the APP protein. As such,
failure of the Peak I β -secretase endoprotease to cleave
35 the Met-MCA bond means that it also does not cleave the
 β -secretase scissile bond. Rather, as discussed below,
25 the majority of the Peak I β -secretase endoprotease
activity preferentially cleaves the Lys-Met in the
40 β -secretase recognition site. Thus, for the Peak I
 β -secretase endoprotease to produce the amino terminal
end of the A β peptide from an APP protein, several
30 cleavages must occur. For example, the Peak I
45 β -secretase endoprotease can cleave the Lys-Met bond
adjacent to the β -secretase scissile bond and, second, an
endogenous β -secretase aminopeptidase can cleave off the
amino terminal Met in the β -secretase scissile bond
50 Met-Asp to produce the amino terminal end of the A β

5 peptide. Alternatively, the Peak I β -secretase
endoprotease can cleave the Val-Lys bond and an
10 endogenous β -secretase aminopeptidase(s) subsequently
cleave off the Lys and Met amino acids and produce the
5 amino terminal end of the A β peptide.

15 In contrast, Peak II was relatively
aminopeptidase insensitive as its activity without
aminopeptidase M was about 84% of that with the
10 aminopeptidase. Thus, the majority of the Peak II
activity cleaves the Met-MCA bond in the substrate
20 Z*Val-Lys-Met-MCA directly because Peak II alone produces
fluorescent free MCA. As the Met-MCA bond is a mimic of
the β -secretase scissile bond, the majority of Peak II
15 β -secretase endoprotease activity also cleaves the
 β -secretase scissile bond which can directly produce the
25 amino terminal end of the A β peptide.

30 But the modest increase in the fluorescence
produced by Peak II with aminopeptidase M indicates that
20 some of the Peak II activity also cleaves the Lys-Met or
the Val-Lys bond in the Z*Val-Lys-Met-MCA substrate for
35 reasons described above regarding Peak I. Similarly,
some of the Peak II activity also can produce the amino
terminal end of the A β peptide by a combination of
25 endoprotease and aminopeptidase cleavages as discussed
40 above regarding Peak I.

45 These results demonstrate that multiple
 β -secretases are involved in producing an A β peptide from
an APP protein.

ISOLATION OF β -SECRETASES FROM PEAK I

The procedure used to isolate the β -secretases from Peak I is diagramed in Figure 10. The Sephacryl S200 column fractions containing the Peak I β -secretase endoprotease activity were pooled (referred to as the "Peak I Sephacryl S200 fraction") and chromatographed on a chromatofocusing Polybuffer Exchange 94 column (PHARMACIA, Piscataway, NJ, referred to here as "CF"). The CF fractions containing the β -secretase endoprotease activity were pooled and concentrated with buffer exchange to 100 mM citric acid-NaOH, pH 4.5, using an AMICON ultrafiltration apparatus equipped with a YM 10 membrane. (referred to as the "Peak I CF fraction" or "CF fraction," see Krieger, T.K. and Hook, V.Y.H., *ibid.*).

The Peak I CF fraction, in turn, was purified using cation Mono S exchange chromatography by FPLC (referred to as "Mono S"). The CF fraction was loaded onto a Mono S ion exchange FPLC column (1 ml HiTrap column SP, PHARMACIA, Piscataway, NJ) that was equilibrated with 100 mM citric acid-NaOH, pH 4.5 (referred to as "buffer A"). The column was eluted with a NaCl gradient generated with a buffer consisting of 100 mM citric acid-NaOH, pH 4.5, 2.0 M NaCl (referred to as "buffer B"), with the gradient consisting of 0% buffer B at 1-15 min., 0-25% buffer B at 15-45 min., 25-100% buffer B at 45-50 min., 100% buffer B at 50-55 min., 100-0% buffer B at 55-60 min., and 0% buffer B at 60-75 min., with a flow rate of 1ml/min. Fractions containing β -secretase endoprotease activity were pooled and concentrated by AMICON ultrafiltration with buffer exchange to 100 mM citric acid-NaOH, pH 4.5 (referred to as the "Peak I Mono S fraction" or "Mono S fraction").

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The Mono S fraction was further analyzed by various polyacrylamide gel electrophoresis (PAGE) methods. Referring in Figure 10, one such method was a "native PAGE in gel activity assay," which determined the β -secretase endoprotease activity of the Mono S fraction in the PAGE gel. In this assay, the proteins are first separated by electrophoresis and then allowed to proteolytically react with a suitable substrate in the gel. Proteins having proteolytic activity are identified by the formation of a cleavage product in the gel. A suitable substrate and cleavage product for detecting a secretase in this assay is an APP substrate and an APP derived product. The APP derived product can be detected by various methods such as those described above, but fluorescent detection methods are preferred. The PAGE in gel activity assay can also be used to detect proteases other than secretases using suitable substrates. The in gel activity assay may also use other suitable gels, such as, for example, agarose. In contrast to the PAGE in gel protein staining assays described below, the PAGE in gel activity assay determines only those proteins having protease activity rather than all proteins.

In a native PAGE in gel activity assay, the sample is in a solution which preserves protein complexes composed of proteins associated together by non-covalent and covalent bonds in their "native" state. Thus, a native PAGE in gel activity assay can determine the proteolytic activity of a protein complex. If a protein complex has such activity, that complex is referred to as a "protease complex." A protease complex is two or more proteins associated together by a non-covalent bond, such as, for example, an ionic bond, or a non-peptide covalent bond, such as, for example, a disulfide bond, and at least one of the proteins has protease activity. A

5 β -secretase protease complex is a protease complex that
cleaves an APP substrate.

10 Referring to Figure 10, another PAGE method
that the Mono S fraction was subjected is the
5 "non-reducing SDS-PAGE in gel activity assay." Like the
native PAGE in gel activity assay, the non-reducing SDS-
15 PAGE in gel activity assay also determined the
 β -secretase endoprotease activity of the Mono S fraction
in the PAGE gel. But this assay differs in that it
20 contains the detergent SDS, hence the term "SDS-PAGE."
SDS separates proteins associated together by a
non-covalent bond. A "non-reducing in gel assay" means
that the assay does not contain a reducing agent, such
25 as, for example, β -mercaptoethanol. Such reducing agents
sever covalent disulfide bonds between and within
15 proteins. Thus, in the non-reducing SDS-PAGE in gel
activity assay, proteins associated by a non-covalent
bond are separated from each other but those proteins
30 that are linked by a disulfide bond are not.

20 The substrate used in all in gel activity
assays was the peptide Z*Phe-Arg-MCA (PENINSULA
35 LABORATORIES, San Carlos, CA). The Phe-Arg-MCA sequence
of that sequence mimics the Val-Lys-Met sequence in the
 β -secretase recognition site because both contain a
40 hydrophobic amino acid adjacent to a positively charged
amino acid and the MCA group, as discussed above, mimics
a P1' amino acid. As such, cleavage of the Arg-MCA bond
in the Z*Phe-Arg-MCA substrate is equivalent to cleaving
45 the Lys-Met bond in the β -secretase recognition site or
in the Z*Val-Lys-Met-MCA substrate. That later substrate
30 was not used for the in gel assay because, as discussed
above, an aminopeptidase is required to detect cleavage
50 of that substrate by Peak 1.

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Native PAGE in gel activity assays were conducted as follows. The Z*Phe-Arg-MCA substrate was embedded into the gel by copolymerization of Z*Phe-Arg-MCA (250 μ M) with resolving gel (8.7 x 0.1 cm, NOVEX gel cassette, San Diego, CA) components consisting of 12% polyacrylamide with 0.16% bis-acrylamide and 0.375 Tris-HCl, pH 8.8. The stacking gel was 6% polyacrylamide, 0.16% bis-acrylamide, and 0.125 M Tris-HCl, pH 6.8, prepared according to Laemmli (Laemmli, U.K. Nature 227:259, 680-685 (1970)). The Mono S fraction (2-4 μ l) was prepared in native sample buffer containing 50 mM Tris-HCl, pH 8.3, and 2% glycerol, and electrophoresed in the gel at 4°C in a running buffer consisting of 25 mM Tris-HCl, 192 mM glycine, pH 8.3 for 2.5 hours at a constant current of 25 mAmp. The gel was then washed in cold 2.5% Triton X-100 solution for 10 minutes, and with cold sterile water for 10 minutes. β -secretase endoprotease cleavage of the substrate Z*Phe-Arg-MCA embedded in the gel was conducted by incubating the gel at 37°C for 2 hours in 100 mM citric acid-NaOH, pH 5.0, 1 mM EDTA, 1 mM DTT, and 10 mM CHAPS. AMC fluorescence in the gel was visualized under a UV transilluminator. The fluorescent image was photographed with Kodak DC120 digital camera, and analyzed with the EDAS120 image software system, which allows quantitative image analysis.

The native PAGE in gel activity assay of the Peak I Mono S fraction resulted in a wide broad band of faint fluorescence. That result is characteristic of a protease complex and shows that the activity in Peak I is due to a protease complex. Moreover, the result shows that the protease complex cleaves the Arg-MCA bond because that cleavage must occur for fluorescence to be

5 detected and fluorescence was detected without an
aminopeptidase being present. Since the Arg-MCA bond in
the Z*Phe-Arg-MCA substrate is equivalent to the Lys-Met
10 bond in the β -secretase recognition site, the protease
5 complex also cleaves the Lys-Met bond in that substrate.

15 The non-reducing SDS-PAGE in gel activity assay
was conducted as described for the native PAGE in gel
activity assay, except that the stacking and resolving
gels contained 0.1% SDS, the sample buffer contained 1.5%
10 SDS, and the electrophoresis was conducted for 1.5 hours.
20 The non-reducing SDS-PAGE in gel activity assay showed 3
distinct, precise and intense fluorescent bands
corresponding to proteins having molecular weights of
approximately 88, 81, and 66 kDa. The 3 proteins cleaved
25 the Arg-MCA bond in the Z*Phe-Arg-MCA substrate because
15 fluorescence was produced without aminopeptidase.
Moreover, those proteins also cleave the Lys-Met bond in
the β -secretase recognition site for the reasons
30 discussed above.

20 The Peak I Mono S fraction was also subjected
35 to "preparative native PAGE." This electrophoresis
method was used to further isolate the β -secretases.
Native conditions using the MiniPrep Cell system (BIORAD,
Richmond, CA). Tube gels (7 mm internal diameter) were
40 25 prepared with the resolving gel (10 cm) consisting of 6%
polyacrylamide (with 0.16% bis-acrylamide and 0.375 M
Tris-HCl, pH 8.8) and a stacking gel (1 cm) of 4%
polyacrylamide (with 0.11% bis-acrylamide and 0.125 M
45 Tris-HCl, pH 6.8), prepared according to the
30 manufacturer's protocol. The Mono S fraction (200 to 300
 μ l) in native sample buffer containing 25 mM Tris-HCl,
192 mM glycine, pH 8.3, and 10% glycerol was subjected to
50 electrophoresis in the native tube gel at a constant

5 power of 1 watt at 4° C for 48 hours in running buffer
consisting of 25 mM Tris-HCl, 192 mM glycine, and pH 8.3.
During electrophoresis, fractions (0.6 ml/fraction) were
10 eluted in running buffer at a flow rate of 0.02
5 ml/minute; stability of eluted β -secretase endoprotease
activity was improved with adjustment of fractions to pH
6.0 using an equal volume of 0.1 M citric acid-NaOH, pH
15 4.5. Fractions were immediately assayed for
Z*Val-Lys-Met-MCA cleavage in the presence of
10 aminopeptidase M, or for Z-Phe-Arg-MCA without
aminopeptidase M as described (Azaryan, A.V. and Hook,
20 V.Y.H., *FEBS Lett.* 341, 197-202 (1994)). After
preparative native gel electrophoresis, one peak of β -
secretase endoprotease activity was observed for cleavage
25 of the substrate Z*Val-Lys-Met-MCA.

The preparative native PAGE sample containing
the activity was further analyzed by various PAGE
30 methods, including the non-reducing SDS-PAGE in gel
20 activity assay described above. That assay resulted in
the same 3 activity bands having molecular weights of
about 88, 81, and 61 kDa obtained from the Mono S
35 fraction run in that assay.

The preparative native PAGE sample was also
25 analyzed in a non-reducing SDS-PAGE in gel protein
40 staining assay which detects the proteins present in the
gel. In contrast to the in gel activity assay, the
protein staining assay detects all proteins present in a
sufficient amount to be detected without regard to
45 protease activity. The non-reducing SDS-PAGE in gel
30 protein staining assay was conducted in a similar manner
as the activity assay, but was silver stained to identify
the proteins and resulted in 3 definite and precise bands
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corresponding to proteins having molecular weights of about 88, 81, and 36 kDa.

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The results obtained from the non-reducing SDS-PAGE in gel protein staining and activity assays were compared. The 88 and 81 kDa proteins observed by silver staining correlated with the two β -secretase endoproteolytic activities at those weights in the activity assay. But no protein was detected in the protein staining assay corresponding to the 61 kDa activity band. This result implied that the amount of protein at that position may have been insufficient to be detected by silver staining. If that is the case, the 61 kDa protein had a very high specific activity because intense activity was observed at that position. No activity was detected in the activity assay at the position corresponding to the 36 kDa protein, indicating that the 36 kDa protein does not have β -secretase endoproteolytic activity.

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The preparative native PAGE sample was further analyzed in a reducing SDS-PAGE in gel protein staining assay. Like the staining assay described above, this assay also detected the proteins present in the gel without regard to proteolytic activity. But since this assay was conducted in the presence of a reducing agent, β -mercaptoethanol, disulfide bonds were severed. The assay was run as described above for the protein staining assay except that the gel and sample buffer contained β -mercaptoethanol. Four proteins having molecular weights of approximately 66, 60, 33, and 29 kDa were detected.

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The reducing SDS-PAGE in gel protein staining assay resulted in more and on average proteins of lower molecular weight than did the corresponding non-reducing

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5 assay. That difference indicates that the preparative
native PAGE sample contained proteins having disulfide
bonds which were severed by the reducing agent to produce
10 a larger number of proteins with lower molecular weights.
5 In particular, the 80 and 81 kDa proteins had such bonds
severed because only lighter proteins were observed under
reducing conditions. The 33 and 36 kDa proteins obtained
15 under reducing and non-reducing conditions may be the
same protein because their weights are similar.

10 The results obtained from the reducing SDS-PAGE
in gel protein staining and the non-reducing SDS-PAGE in
gel activity assays were compared. The 88 and 81 kDa
proteins having activities contained one or more
25 disulfide bonds that were severed under the reducing
15 conditions. The 60 kDa and 61 kDa proteins in silver
staining and activity assays were about the same weight
and may be the same protein.

30 ISOLATION OF β -SECRETASES FROM PEAK II

20 The procedure used to isolate Peak II-A and
Peak II-B from Peak II is diagramed in Figure 11. The
35 Sephacryl S200 fractions containing Peak II were pooled
and further purified using Mono Q ion exchange FPLC
chromatography (referred to as "Mono Q FPLC"). The
40 25 fraction that did not bind to that column contained Peak
II β -secretase endoprotease activity (referred to as the
"unbound Peak II" or "Peak II-A"). The fraction that
bound to the column was eluted using a NaCl gradient from
45 zero to 0.5 M NaCl, and also contained Peak II
30 β -secretase endoprotease activity (referred to as "bound
Peak II" or the "Peak II-B"). Peak II-B was further
purified by a second Mono Q column, with elution of the
50 β -secretase activity by a pH gradient of pH 7.0 to pH 4.0

5 generated by polybuffer 74 (PHARMACIA, Piscataway, NJ),
performed as described previously (Krieger, T.K. and
Hook, V.Y.H., *ibid.*). Since Mono Q FPLC is an anion
10 exchange chromatography, the unbound Peak II is a protein
5 that is less electronegative than the Peak II-B protein.

- EXAMPLE XIII

15 β -secretase Endoprotease Activities Obtained During
Isolation of β -secretases

20 The total (relative fluorescence units/0.5 hr)
10 and specific (relative fluorescence units/mg protein) of
the β -secretase endoprotease activity without and with
aminopeptidase M (-APM, +APM, respectively) was
25 determined for fractions obtained in the isolation
procedure described in Example XII. All assays were
15 conducted as described in Example IX. The activities
obtained are summarized in Table III.

TABLE III

ISOLATION STEP	TOTAL ACTIVITY		SPECIFIC ACTIVITY	
	-APM	+APM	-APM	+APM
Lysate	11	12	1.8	1.9
Soluble extract	12	12	2.6	2.5
Membrane	0.4	0.6	1.7	2.3
Con-A bound ^a	19	75	367	1.5×10^3
Con-A unbound ^b	8	9	2	2
Peak I				
Sephacryl S200	13	275	2.0×10^3	4.2×10^4
CF fraction	38	496	3.0×10^3	3.8×10^4
Mono S fraction	16	300	5.0×10^5	9.3×10^6
Prep. SDS-PAGE	ND ^c	30	1.0×10^7	2.0×10^7
Peak II				
Sephacryl S200	63	75	6.0×10^4	7.2×10^4
Mono Q FPLC				
Peak II-A	15	16	5.5×10^5	6.0×10^5
Mono Q FPLC				
Peak II-B	6	6	3.1×10^4	4.4×10^4
^a No β -secretase aminopeptidase activity detected				
^b β -secretase aminopeptidase activity detected				
^c Not done				

The total activity of the lysate and the soluble extract without aminopeptidase M was about 92% and 100% of that with the aminopeptidase, respectively, and thus were aminopeptidase insensitive. The soluble extract contained about 100% of the total activity in the lysate, but the membrane pellet contained only about 4%

5 of that activity, indicating that the β -secretase
endoprotease activity is not bound to the chromaffin
vesicle membranes.

10 The eluted Con-A bound fraction assayed without
5 and with aminopeptidase M had about 158% and 625% of the
total activity in the lysate, respectively. The increase
15 in the total activity indicated that an inhibitor or
competitive substrate, such as APP protein, may be
removed at this step. The eluted Con-A bound fraction
10 had a total activity that was somewhat aminopeptidase
sensitive as the activity without aminopeptidase M was
20 approximately 25% of that with the aminopeptidase.

25 The Con-A unbound fraction contained the
endogenous β -secretase aminopeptidase activity which was
15 not present in the eluted Con-A bound fraction. As such,
Peak I and Peak II subsequently purified from the eluted
Con-A bound fraction did not contain significant
30 endogenous aminopeptidase activity.

35 Peak I from the Sephacryl S200 isolation step
20 was highly aminopeptidase sensitive, having a total
activity of only about 4.7% without aminopeptidase M as
and with the aminopeptidase. Moreover, Peak I assayed
40 with the aminopeptidase had about 367% and 2292% of the
total activity in the eluted Con-A bound fraction and
25 lysate, respectively, again indicating possible removal
of an inhibitor or competitive substrate.

45 Continuing with the isolation of Peak I, the CF
fraction also was aminopeptidase sensitive as the total
activity without aminopeptidase M was about 7.6% of that
30 with the aminopeptidase. Again the total activity was
50 increased, this time by about 180% and 4,133% of that

5 from the Sephacryl S200 fraction and the lysate,
respectively, as measured with aminopeptidase M and again
10 raising the possibility that an inhibitor or competitive
substrate was removed.

5 The Mono S fraction of Peak I remained very
aminopeptidase sensitive, having a total activity without
15 aminopeptidase M of about 5.3% of that with the
aminopeptidase. But the total activity of the Mono S
fraction was about 60% and 2,500% of that in the CF
10 fraction and lysate, respectively. This indicates that
the Mono S isolation step may lose some activity but that
20 the activity remains well above that in the lysate.

25 The preparative SDS-PAGE isolation of Peak I
15 resulted in 10% and 250% of the activity in the Mono S
fraction and lysate, respectively. Moreover, the
activity after this step, unlike the previous isolation
steps, became quite unstable indicating that the
30 preparative SDS-PAGE isolation step may remove an
activator or stabilizing agent.
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35 Returning to the isolation of Peak II by
Sephacryl S200, the Peak II had about 27% of the activity
of Peak I. In other words, Peak I had about 3 times more
 β -secretase endoprotease activity than did Peak II. But
40 25 Peak II was relatively aminopeptidase insensitive as the
total activity without aminopeptidase M was about 84% of
that with the aminopeptidase. Peak II total activity
assayed with aminopeptidase M was the same as that in the
45 eluted Con-A bound fraction indicating that this
30 isolation step does not remove an inhibitor, an APP
substrate, an activator, or a stabilizing agent.

5 After Mono Q FPLC isolation, Peak II-A and Peak
II-B were found to be aminopeptidase insensitive. The
combined total activity of Peak II-A and Peak II-B was
10 about 32% of the total activity in the Sephacryl S200
5 fraction with aminopeptidase M. Peak II-A and Peak II-B
had a total activity of about 133% and 66% of that in the
lysate, respectively. -

15 The specific activity showed that a very high
degree of isolation was obtained. Specifically, the
10 preparative SDS-PAGE electrophoresis isolation step of
Peak I resulted in about a 0.5×10^6 and 1.0×10^6
purification from the chromaffin vesicle lysate as
analyzed without and with aminopeptidase, respectively.
The Mono Q FPLC isolation of Peak II-A resulted in a 2.3
25 $\times 10^5$ and 3×10^5 purification from the chromaffin vesicle
lysate as analyzed without and with aminopeptidase,
respectively. The Mono Q FPLC isolation step of the
Peak II-B resulted in a 1.5×10^4 and 2.2×10^4
30 purification from the chromaffin vesicle lysate as
20 analyzed without and with aminopeptidase, respectively.

35 EXAMPLE XIV

Protease Inhibitors of β -secretase Endoprotease Activity
in Peaks I and II

40 The effect of various protease inhibitors on
25 β -secretase endoprotease activity in Peaks I and II was
determined by the method described in Example XI. The
results were expressed as a percent inhibition of the
45 control (no inhibitor) is summarized in Table IV.

TABLE IV

PROTEASE CLASS	INHIBITOR (Concentration)	Peak I (%)	Peak II (%)
Control	None	100	100
5 Cysteine	E64c (10 μ M)	0	0
15 Cysteine	pHMB (1 mM)	67	68
Serine	PMSF (100 μ M)	90	112
Serine	Chymostatin (10 μ M)	0	35
Aspartyl	Pepstatin A (100 μ M)	85	132
20 Metallo	EDTA (1 mM)	99	138
Metallo	EGTA (1 mM)	108	142
Metallo	1,10 Phenanthroline (500 μ M)	31	72
25 Nonspecific	Leupeptin (100 μ M)	0	0

Peak I and Peak II activities were maximally inhibited by the nonspecific protease class inhibitor leupeptin, the cysteine class inhibitor E64c, and the serine protease class inhibitor chymostatin. The other cysteine class inhibitor, pHMB, slightly inhibited both activities. The other serine protease class inhibitor, PMSF, did not significantly inhibit either activity. The metallo protease class inhibitor 1,10 phenanthroline significantly inhibited Peak I, but only slightly inhibited Peak II. The other metallo protease class inhibitors and the aspartyl protease class inhibitor pepstatin A did not significantly inhibit either activity.

Peak I and Peak II activities were identically inhibited by the cysteine protease class and nonspecific protease class inhibitors. The serine, aspartyl and

5 metallo protease classes inhibitors tended to inhibit
Peak I activity more than Peak II.

10 The inhibition of Peak I and Peak II activities
was compared with that obtained for the chromaffin
5 vesicle lysate (Example XI). All 3 activities were
completely inhibited by the cysteine protease class
15 inhibitor E64c and the nonspecific protease class
inhibitor leupeptin. The serine protease class inhibitor
chymostatin and the cysteine protease class inhibitor
10 pHMB inhibited all activities, although the Peak I and
Peak II activity was inhibited less than that of the
lysate. The serine protease class inhibitor PMSF
25 significantly inhibited the lysate activity but only
slightly inhibited the Peak I and Peak II activities.
15 The aspartic protease class inhibitor pepstatin A
slightly inhibited the lysate and Peak I activities but
increased the activity of Peak II. Except for the 1,10
30 phenanthroline, none of the metallo class protease class
inhibitors inhibited any activity and, in some cases,
20 increased the activity.

35 EXAMPLE XV

Confirmation of Cleavage Specificities of the Peak I,
Peak II-A, and Peak II-B β -Secretase Endoprotease
Activities

40 As discussed above, the substrates
25 Z-Val-Lys-Met-MCA and Z-Phe-Arg-MCA mimic the β -secretase
recognition site in the APP protein. The fluorescent MCA
45 that resulted from the cleavage of those substrates
established the cleavage specificities of the Peak I,
30 Peak II-A, and Peak II-B β -secretases. In particular,
those results showed that the majority of the
50 endoprotease activity in Peak I cleaved the Lys-Met bond

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amino terminally adjacent to the β -secretase scissile bond in the β -secretase recognition site of the APP protein. Those results also showed that the majority of the endoprotease activity in Peak II-A and Peak II-B

5 cleaved the β -secretase scissile bond in the β -secretase recognition site of the APP protein.

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To confirm the Peak I cleavage specificity, electrospray mass spectrometry (EMS) was also used to analyze the APP derived products resulting from the

10 cleavage of the Z*Val-Lys-Met-MCA substrate by the Peak I activity. The cleavage assay was conducted by the method described in Example XII without aminopeptidase M. The APP derived products were then analyzed by a commercial EMS facility (SCRIPPS RESEARCH INSTITUTE, La Jolla, CA).

15 The EMS analysis confirmed that the Peak I activity cleaved the Lys-Met bond in the Z*Val-Lys-Met-MCA substrate.

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To confirm the cleavage specificities of the Peak I, Peak II-A, Peak II-B activities, another APP

20 substrate was reacted with each of those activities and the APP derived products analyzed by EMS. The APP substrate Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe (SEQ ID NO.:5) contains the 5 amino terminal and 4 carboxyl

terminal amino acids to the β -secretase scissile bond in

25 the APP protein. The substrate was commercially produced and purified to greater 95% purity by standard reverse phase high pressure liquid chromatography methods. The cleavage assay of Example XII was used without the

aminopeptidase M and without the Z*Val-Lys-Met-MCA

30 substrate, but with the Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe (SEQ ID NO.:5) substrate (14 μ g/assay). The APP derived products were

then subjected to a C8 reverse phase high pressure liquid

5 chromatography, eluted with an acetonitrile gradient in
0.1 % TFA (trifluoroacetic acid), the peptides identified
by absorbance spectroscopy at 210-215 nm and collected
10 (see Krieger T.K. and Hook V.Y.H., *ibid.* and Krieger et
5 al., *J. Neurochem.* 59, 26-31 (1992)). The EMS data of
the eluted APP derived products confirmed that the
majority of Peak I activity cleaved the Lys-Met bond and
15 that the majority of the Peaks II-A and II-B activities
cleaved the Met-Asp bond.

10

The above-identified references are expressly
20 incorporated herein. Although the invention has been
described with reference to the examples provided above,
it should be understood that various modifications can be
15 made without departing from the spirit of the invention.
25 Accordingly, the invention is limited only by the claims.

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Claims

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I claim:

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1. A method of determining the proteolytic activity of a secretase, comprising the steps of:

- 5 a) obtaining substantially pure vesicles;
b) permeablizing the substantially pure vesicles;

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c) incubating the permeablized vesicles with an APP substrate; and

20

d) detecting the cleavage of the APP substrate, wherein the amount of cleavage is proportional to the proteolytic activity of the secretase.

25

2. The method of claim 1, wherein the secretase is selected from the group consisting of β -secretase, γ -secretase, and α -secretase.

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15 3. The method of claim 1, wherein the vesicles are secretory vesicles.

4. The method of claim 1, wherein the vesicles are chromaffin vesicles.

35

5. The method of claim 1, wherein the APP substrate is selected from the group consisting of an APP protein, an A β peptide and a peptide containing a secretase recognition site.

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6. The method of claim 5, wherein the peptide is selected from the group consisting of Lys-Met, Val-Lys-Met, Val-Val, Gly-Val-Val, Ile-Ala, Val-Ile-Ala, Ala-Thr, and Ile-Ala-Thr.

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7. The method of claim 5, wherein the peptide further contains one or more blocking groups.

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8. The method of claim 7, wherein the peptide contains an amino terminal blocking group.

10

9. The method of claim 7, wherein the peptide contains a carboxyl terminal blocking group.

15

10. The method of claim 7, wherein the peptide contains an amino terminal and carboxyl terminal blocking group.

20

11. The method of claim 10, wherein:

a) the peptide is selected from the group consisting of Lys-Met, Val-Lys-Met, Val-Val, Gly-Val-Val, Ile-Ala, Val-Ile-Ala, Ala-Thr, and Ile-Ala-Thr;

25

b) the amino terminal blocking group is selected from the group consisting of acyl (Ac), benzoyl (Bz), succinyl (Suc), carbobenzoxy (Z),

30

15 *p*-bromocarbobenzoxy, *p*-chlorocarbobenzoxy, *p*-methoxycarbobenzoxy, *p*-methoxyphenylazocarbobenzoxy, *p*-nitrocarbobenzoxy, *p*-phenylazocarbobenzoxy, tert-butoxycarbonyl (Boc), and benzoyl; and

35

c) the carboxyl terminal blocking group is selected from the group consisting of aminomethylcoumarinamide (MCA), the diazomethanes, the *p*-nitroanalide (pNA), pNA-Tosylate, 2-naphthylamine, the acyloxymethanes, including the (benzoyloxy)methanes, (alkyloxy)methanes, the N,O-diacyl hydroxamates, including the N-aminoacyl-O-4-nitrobenzoyl hydroxamates, esters, including methyl, ethyl and nitrophenyl esters, and chloromethylketone.

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12. The method of claim 10, further comprising adding an aminopeptidase to the incubation step.

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13. The method of claim 1, wherein the incubating step is conducted at a pH of about 7.0 or less.

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14. The method of claim 1, wherein:

- a) the secretase is a β -secretase;
- b) the vesicles are chromaffin vesicles;
- c) the APP substrate is Z*Val-Lys-Met-MCA;
- d) the incubating step is initially conducted

15

10 at a pH of about 7.0 or less;

20

e) the pH is raised to above pH 7.0;

f) an aminopeptidase is added to the incubation solution; and

25

15 e) the cleavage is detected by the fluorescence of free MCA which is proportional to proteolytic activity of the β -secretase.

30

15. A method of isolating a secretase, comprising the steps of:

- a) obtaining substantially pure vesicles;
- b) permeablizing the substantially pure vesicles;

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c) incubating the permeablized vesicles with an APP substrate;

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d) detecting the cleavage of the APP substrate, wherein the amount of cleavage is proportional to the proteolytic activity of the secretase; and

e) isolating the secretase having the activity.

45

16. The method of claim 15, wherein the secretase is selected from the group consisting of the β -secretase, γ -secretase, and α -secretase.

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17. The method of claim 15, wherein the vesicles are secretory vesicles.

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18. The method of claim 15, wherein the vesicles are chromaffin vesicles.

5

19. A secretase produced by the method of claim 15.

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20. A method of selecting an agent that alters the cleavage of an APP substrate by a secretase, comprising the steps of:

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a) obtaining substantially pure vesicles;

b) permeablizing the substantially pure

vesicles;

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c) incubating the permeablized vesicles with an APP substrate in a first incubation solution;

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d) detecting the cleavage of the APP substrate in the first incubation solution, wherein the amount of cleavage is proportional to the proteolytic activity of the secretase;

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e) incubating the agent, the permeablized

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20 vesicles, and the APP substrate in a second incubation solution;

f) detecting the cleavage of the APP

substrate in the second incubation solution, wherein the

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amount of cleavage is proportional to the proteolytic

25 activity of the secretase;

g) comparing the cleavage of the APP

substrate in the first and second incubation solutions;

45

and

h) selecting the agent that alters the

30 cleavage of the APP substrate of the second incubation

solution from that in the first incubation solution.

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21. The method of claim 20, wherein the secretase is selected from the group consisting of β -secretase, γ -secretase, and α -secretase.

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22. The method of claim 20, wherein the vesicles are secretory vesicles.

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23. The method of claim 20, wherein the vesicles are chromaffin vesicles.

20

24. An agent selected by the method of claim 20.

25

25. A method of selecting an agent that alters the cleavage of an APP substrate by a secretase, comprising the steps of:

30

a) obtaining substantially pure vesicles;
b) permeablizing the substantially pure vesicles;

35

c) incubating the permeablized vesicles with an APP substrate;
d) detecting the cleavage of the APP substrate, wherein the amount of cleavage is proportional to the proteolytic activity of the secretase;

40

e) isolating the secretase having the activity;

45

f) incubating the secretase that is isolated with the APP substrate in a first incubation solution;
g) detecting the cleavage of the APP substrate in the first incubation solution, wherein the amount of cleavage is proportional to the proteolytic activity of the secretase that is isolated;

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h) incubating the secretase that is isolated, the APP substrate and the agent in a second incubation solution;

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i) determining the cleavage of the APP substrate in the second incubation solution, wherein the amount of cleavage is proportional to the proteolytic activity of the secretase that is isolated;

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j) comparing the cleavage of the APP substrate in the first and second incubation solutions; and

15

k) selecting the agent that alters the cleavage of the APP substrate in the second incubation solution from that in the first incubation solution.

20

26. The method of claim 25, wherein the secretase is selected from the group consisting of β -secretase, γ -secretase, and α -secretase.

25

27. The method of claim 25, wherein the vesicles are secretory vesicles.

30

28. The method of claim 25, wherein the vesicles are chromaffin vesicles.

35

29. An agent selected by the method of claim 25.

40

30. A method of selecting an agent that alters the production by a cell of a product consisting of APP protein and APP derived product, comprising the steps of:

a) selecting the cell by determining a proteolytic activity of a secretase in vesicles of the cell;

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b) culturing the cell in a first culture solution;

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d) detecting the product in the first culture solution, wherein the amount of product is proportional to the proteolytic activity of the secretase in the cell;

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e) incubating the agent and the cell in a second culture solution;

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f) detecting the product in the second culture solution, wherein the amount of product is proportional to the proteolytic activity of the secretase in the cell;

15

g) comparing the product produced in the first and the second culture solutions; and

h) selecting the agent that alters the production of the product in the second culture solution from that of the first culture solution.

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31. The method of claim 30, wherein the product is selected from the group consisting of an A β peptide, an α -APP fragment and a 10 kDa fragment.

32. The method of claim 30, wherein the cell is a chromaffin cell.

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33. The method of claim 30, wherein the cell in the first and second culture solution is producing the product.

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34. An agent selected by the method of claim 30.

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35. A substantially pure β -secretase comprising a protein having a molecular weight selected from the group consisting of about 61, 81 and 88 kiloDaltons (kDa) as determined by cleavage of an APP substrate in a non-reducing SDS-PAGE in gel activity assay.

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36. The β -secretase of claim 35, wherein the protein cleaves the APP substrate at a β -secretase recognition site.

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37. The β -secretase of claim 36, wherein the protein cleaves the β -secretase recognition site at a bond selected from the group consisting of Lys-Met and Val-Lys.

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38. The β -secretase of claim 37, wherein the protein cleaves the Lys-Met bond.

10

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39. A method of selecting an agent that inhibits a cleavage of an APP substrate comprising contacting the agent with the β -secretase of claim 35 and selecting the agent that inhibits the cleavage of the APP substrate by the β -secretase.

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40. A method of inhibiting production of an A β peptide by a cell comprising contacting the cell with the agent selected by the method of claim 39 and thereby inhibiting production of the A β peptide by the cell.

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41. A method of inhibiting production of an A β peptide by an Alzheimer's disease patient comprising administering to the patient the agent selected by the method of claim 39 and thereby inhibiting production of the A β peptide by the Alzheimer's disease patient.

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42. A substantially pure protease complex having a molecular weight between about 180 and 200 kDa as determined by Sephacryl chromatography that cleaves an APP substrate.

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43. The protease complex of claim 42, wherein the protease complex cleaves the APP substrate at a β -secretase recognition site.

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44. The protease complex of claim 43, wherein the protease complex cleaves the β -secretase recognition site at a bond selected from the group consisting of Lys-Met and Val-Lys.

15

45. The protease complex of claim 44, wherein the protease complex cleaves the Lys-Met bond.

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46. The protease complex of claim 42, further comprising proteins having a molecular weight selected from the group consisting of about 66, 60, 33 and 29 kDa as determined by a reducing SDS-PAGE in gel protein staining assay.

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47. The protease complex of claim 42, further comprising proteins having a molecular weight selected from the group consisting of about 61, 81 and 88 kDa as determined by cleavage of an APP substrate in a non-reducing SDS-PAGE in gel activity assay.

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48. A method of selecting an agent that inhibits a cleavage of an APP substrate comprising contacting the agent with the protease complex of claim 42 and selecting the agent that inhibits the cleavage of the APP substrate by the protease complex.

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49. A method of inhibiting production of an A β peptide by a cell comprising contacting the cell with the agent selected by the method of claim 48 and thereby inhibiting production of the A β peptide by the cell.

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50. A method of inhibiting production of an A β peptide by an Alzheimer's disease patient comprising administering to the patient the agent selected by the method of claim 48 and thereby inhibiting production of the A β peptide by the Alzheimer's disease patient.

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51. A substantially pure β -secretase having a molecular weight between about 50 and 90 kDa as determined by Sephacryl chromatography and that cleaves an APP substrate.

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52. The β -secretase of claim 51, wherein the protease complex cleaves the APP substrate at a β -secretase recognition site.

25

53. The β -secretase of claim 52, wherein the β -secretase cleaves the β -secretase recognition site at a bond selected from the group consisting of Met-Asp, Lys-Met and Val-Lys.

30

54. The β -secretase of claim 53, wherein the β -secretase cleaves the Met-Asp bond.

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55. The β -secretase of claim 51, further comprising 2 proteins having different electronegative charges as determined by ion exchange chromatography.

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56. A method of selecting an agent that inhibits a cleavage of an APP substrate comprising contacting the agent with the β -secretase of claim 51 and selecting the agent that inhibits the cleavage of the APP substrate by the β -secretase.

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57. A method of inhibiting production of an A β peptide by a cell comprising contacting the cell with the

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agent selected by the method of claim 56 and thereby
inhibiting production of the A β peptide by the cell.

10

58. A method of inhibiting production of an A β
peptide by an Alzheimer's disease patient comprising
5 administering to the patient the agent selected by the
method of claim 56 and thereby inhibiting production of
15 the A β peptide by the Alzheimer's disease patient.

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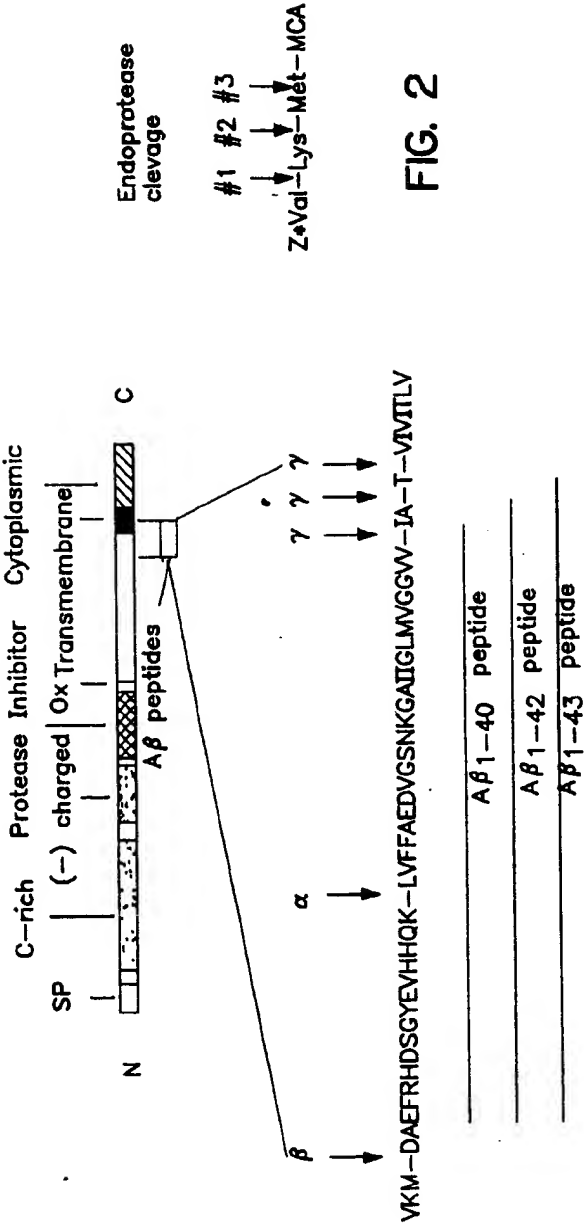


FIG. 2

FIG. 1

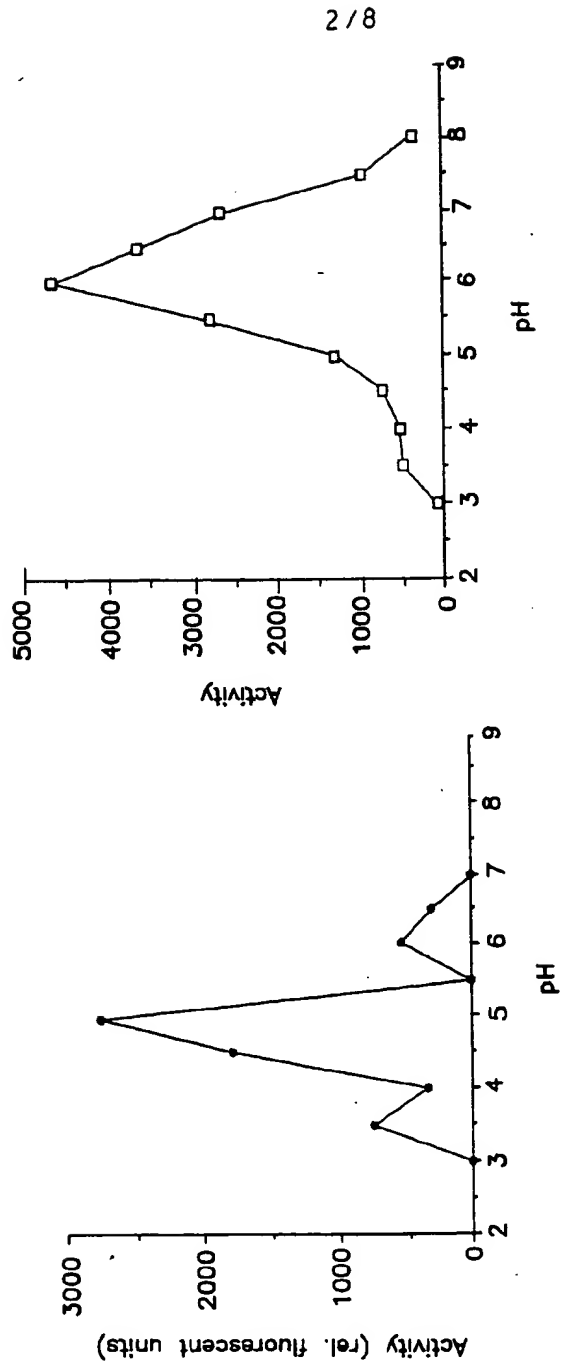


FIG. 4

FIG. 3

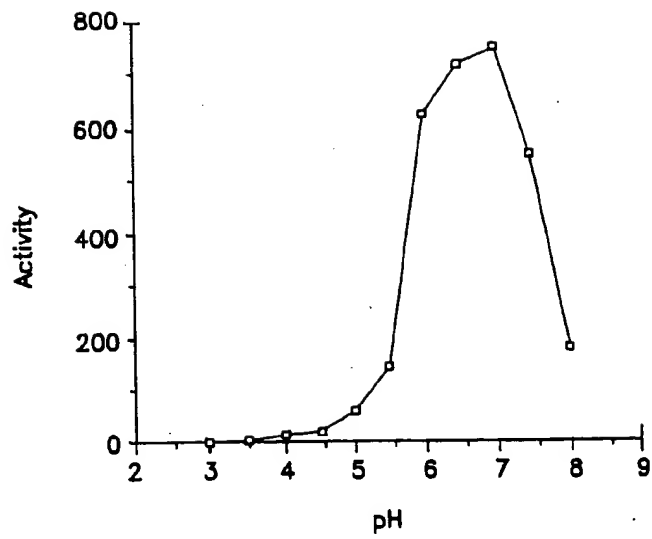


FIG. 5

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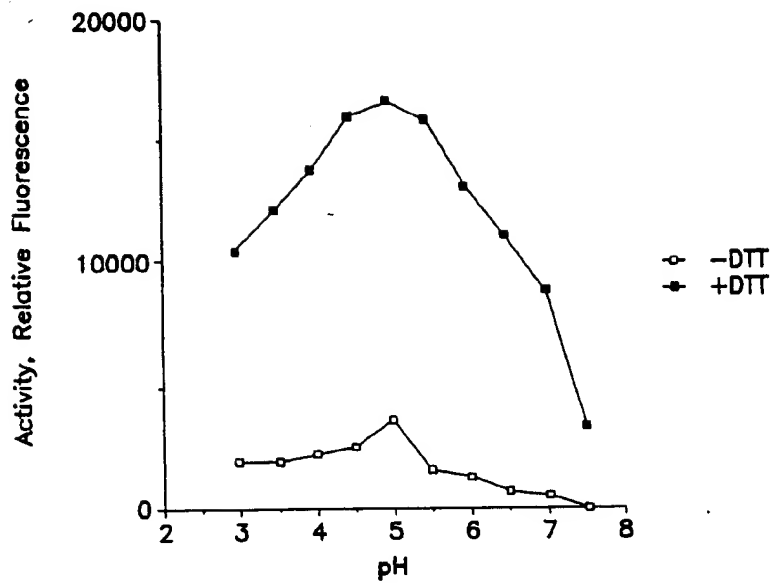


FIG. 6

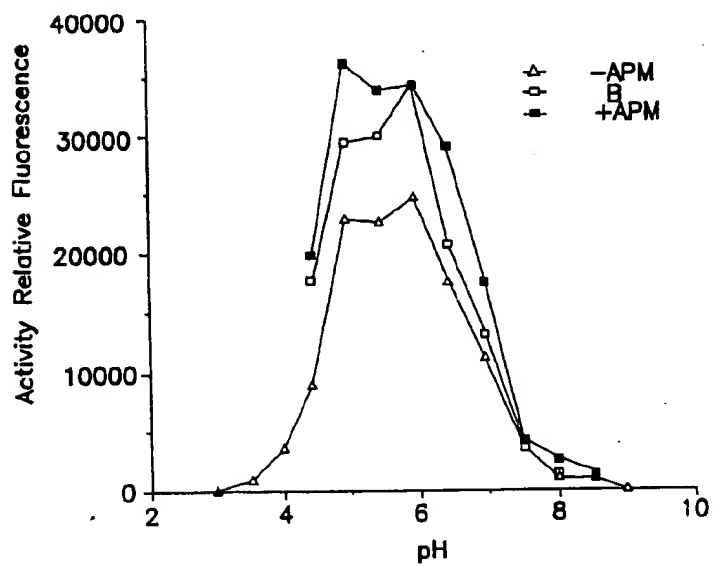


FIG. 7

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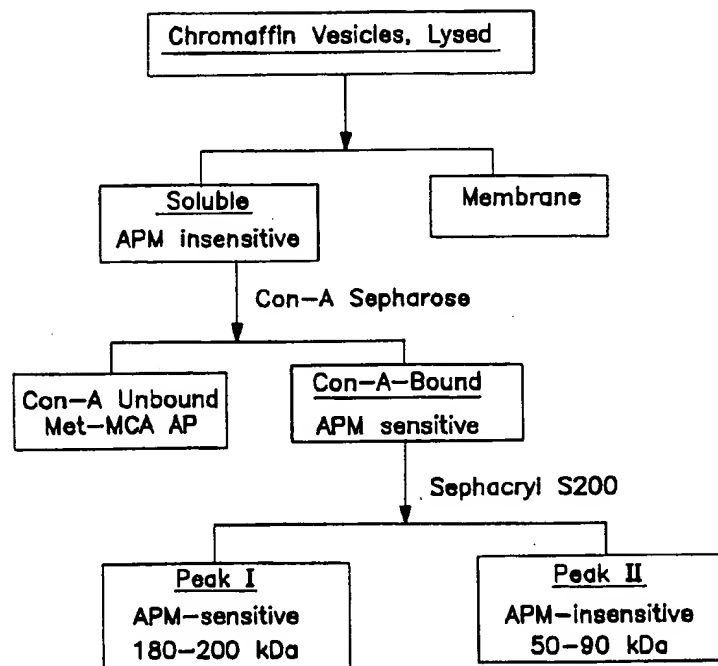


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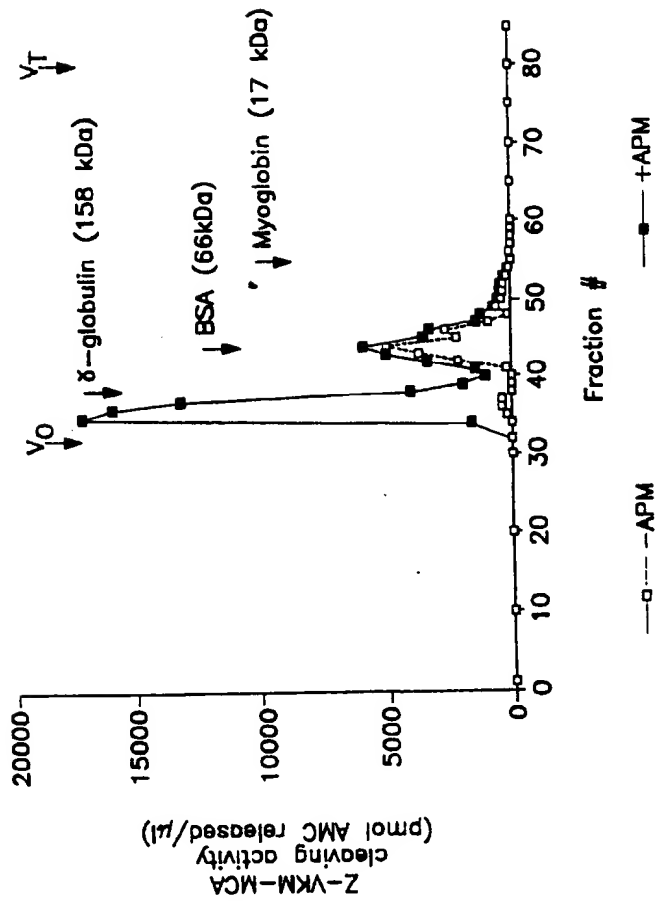


FIG. 9

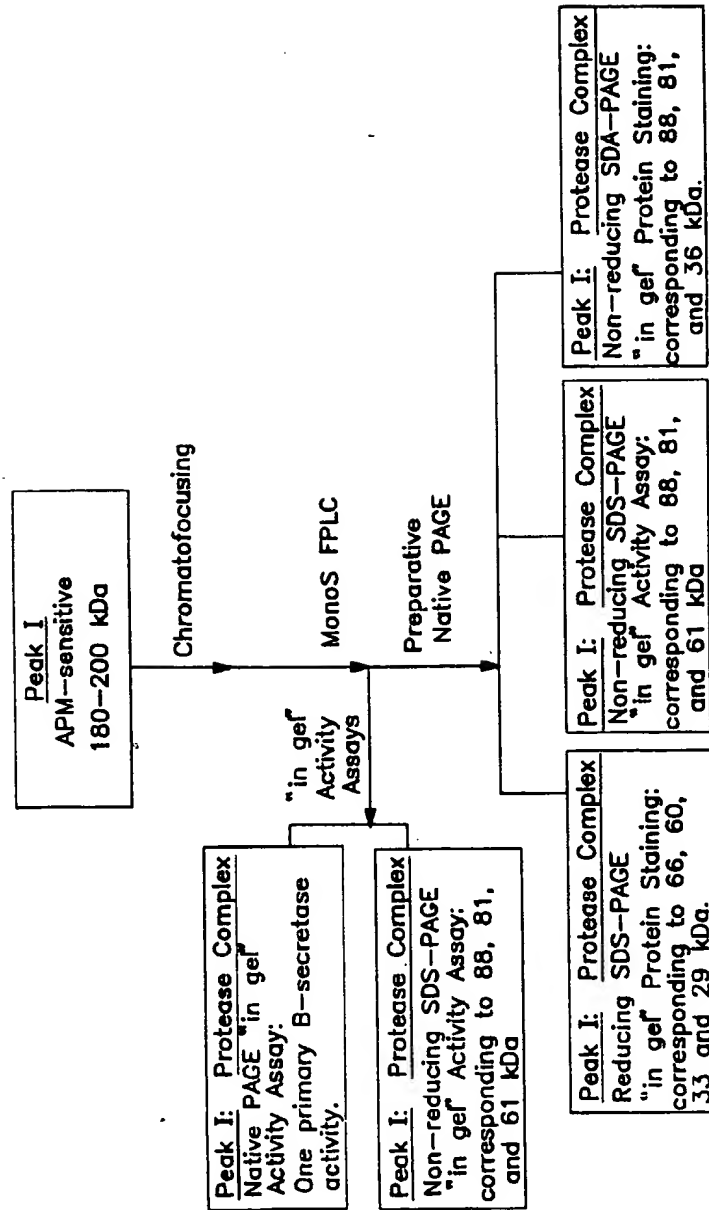


FIG. 10

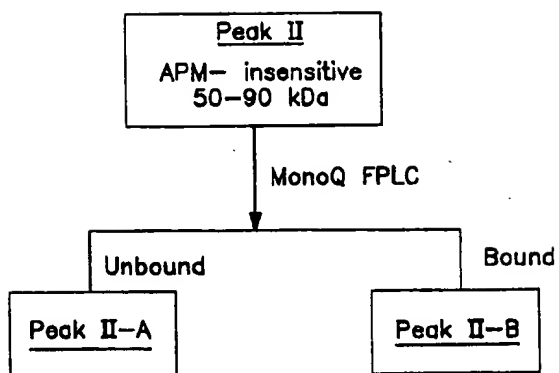


FIG. II

SEQUENCE LISTING

<110> Hoox, Vivian Y.H.
ActiveSite Biotech

<120> SECRETASES RELATED TO ALZHEIMER'S DEMENTIA

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<150> US 09/173,887

<151> 1998-10-16

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<151> 1999-04-20

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			20					25					30		

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WO 00/23576

PCT/US99/24403

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